IDENTIFICATION AND CHARACTERIZATION OF *SHORT VEGETATIVE PHASE* (*SVP*) TARGET GENES

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List of Abbreviations

Chemicals and Reagents

DEPC	diethylpyrocarbonate
dNTP	deoxynucleoside triphosphate
EDTA	ethylene-diamine-tetra-acetate
Gly	glycine
HCl	hydrochloric acid
KPO_4	potassium phosphate
LB broth	Luria-Bertani broth
LiCl	lithium chloride
$MgCl_2$	magnesium chloride
NaCl	sodium chloride
Na_2HPO_4	disodium phosphate
NaH_2PO_4	sodium phosphate
PBS	phosphate buffered saline
PMSF	phenylmehtylsulfonylfluoride
PVA	polyvinyl alcohol
SDS	sodium dodecylsulphate
Tris	tris-(hydroxymethyl)aminomethane

Units and Measurements

bp	base pair(s)
g	gram(s)
hr	hour(s)
kb	kilo base-pair(s)
kDa	kilo Dalton(s)
M	molar
min	minute(s)
ml	mililitre(s)
mM	milimolar
ng	nanogram(s)
OD_{600nm}	absorbance at wavelength 600 nm
rpm	revolutions per minute
sec	second(s)
U	unit(s)
v / v	volume per volume
w / v	weight per volume
°C	degree Celsius
µg	microgram(s)
µl	microlitre(s)
µM	micromolar

Others

amiRNA	artificial micro ribonucleic acid
BLAST	Basic Local Alignment Search Tool
cDNA	complementary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
cRNA	complementary ribonucleic acid
CDK	cyclin-dependent kinase
Col	Columbia
DNA	deoxyribonucleic acid
et al.	et alter (and others)
GA	gibberellin, or gibberellic acid
i.e.	that is
LD	long day
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SAM	shoot apical meristem
SD	short day

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Summary

Flowering plants undergo floral transitions from vegetative phase to reproductive phase in response to multiple endogenous and environmental signals. In *Arabidopsis, SHORT VEGETATIVE PHASE* (*SVP*) has been suggested as a central regulator of flowering time. Recent findings have indicated that SVP functions by interacting with FLC to control the transcription of two floral pathway integrators, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *FLOWERING LOCUS T* (*FT*). In a search for novel target genes of *SVP* that mediate its function in flowering regulation, we identified that *AtPIN1* was transcriptionally regulated by SVP and that it promoted flowering under both long days and short days. *AtPIN1* responds to both photoperiod and vernalization, and its function as a flowering promoter depending on the activity of SOC1 and AGL24 was revealed by genetic cross analysis. In addition, this interaction between AtPIN1 and SOC1/AGL24 occurred at post-transcriptional level. Our data suggest that, as an enzyme that catalyzes *cis*/*trans* conformation change, AtPIN1 may bind to SOC1 and AGL24 and facilitates their conformational change, leading to the accumulation of specific conformations of these two proteins to promote flowering.

Chapter 1 Literature Review

1.1 Introduction

Flowering plants, also known as angiosperms, are the most successfully evolved and predominant group of land plants, characterized by their most remarkable feature, i.e. flowers. They represent the most widespread group of land plants and one of the only two extant groups of seed plants on the planet earth (Magallón et al., 1999). They are easily distinguished from other seed plants by their extremely diversified flower morphologies. Flowering plants serve as the major basis for agriculture through livestock feed, and offer other economic resources as well, including wood, paper, fiber, and medicines, etc. Estimation of their number of species has been made to be in the range of 250,000 to 400,000 (Govaerts, 2001; Govaerts, 2003; Scotland and Wortley, 2003; Thorne, 2002). The reproductive successes of flowering plants depend heavily on the correct timing to switch from vegetative to reproductive phase, which allow plants to flower under desirable conditions for optimal seed setting and synchronously for out-breeding species (Bernier, 1988). This major developmental transition is tightly controlled by an integrated network of pathways that respond to both environmental and endogenous signals and distinct strategies for reproduction have been evolved in different plant species (Simpson and Dean, 2002).

The last 20 years have seen an explosion of knowledge on the molecular and genetic mechanisms underlying floral induction, patterning and organ identity. Three dicot species, *Antirrhinum majus*, *Arabidopsis thaliana*, and *Petunia hybrida* have been the primary sources from which the basic mechanisms are elucidated. Among these three model plants, *Arabidopsis thaliana* is most contributive in giving detailed and comprehensive knowledge about the fundamental molecular mechanisms of flower development (Jack, 2004). *Arabidopsis thaliana* is a small weed in the mustard family under the genus *Brassica* and is native to Europe, Asia, and Northwestern Africa. Its adoption as a genetic model organism was first proposed by Laibach in 1943 based on his findings of the short generation time, fecundity, ease of crosses, and the possibility of mutagenesis for *Arabidopsis* (Laibach, 1943). It was later studied in detail by Rédei in the United States whose instrumental reviews helped introduce the model to the scientific community (Rédei, 1975). Further momentum for the use of *Arabidopsis* as a model organism came from the release of the first complete and detailed genetic linkage map of *Arabidopsis* (Koornneef et al., 1983), the summarization of the value of *Arabidopsis* as a model system for research in plant biology, the demonstration that its small genome is amenable to detailed molecular analysis (Meyerowitz and Pruitt, 1985), and the significant technical advances leading to the establishment of transformation protocols (An et al., 1986; Feldmann and Marks, 1987; Lloyd

et al., 1986).

The increased enthusiasm for *Arabidopsis* led to the drafting of a vision statement in 1990, which outlined the long-term objectives for the *Arabidopsis* community, and the establishment of the *Arabidopsis* Genome Initiative in 1996 to coordinate the multinational endeavor of the large-scale sequencing of *Arabidopsis thaliana* genome (Meinke et al., 1998). The sequencing started in 1996 and was finished in 2000, but more work is still being done to integrate all available experimental data on gene structure and function into the genome annotation (Swarbreck et al., 2008; The Arabidopsis Genome Initiative, 2000). The estimated ~157Mb genome of *Arabidopsis thaliana*, which is organized into five chromosomes, contains 27,235 protein coding genes, 4,759 pseudo genes or transposable elements and 1288 non-coding RNAs (ncRNAs) (33,282 genes in all, 38,963 gene models) according to the newest gene annotation released from the Arabidopsis Information Resource, TAIR8 (Bennett et al., 2003; The Arabidopsis Genome Initiative, 2000). The availability of the whole genome sequence of *Arabidopsis* changed the nature of plant genetic research fundamentally, making forward genetics greatly simplified and reverse genetics possible. The meteoric rise of *Arabidopsis thaliana* as a model organism from an obscure weed represents not only an integration of scattered community resources, avoiding duplication of effort and waste of funding, but also a dramatic shift in paradigm for plant biology research (Meinke et al.,

1998). In the year 1998, *Arabidopsis thaliana* has officially been selected as one of the members of "Security Council of Model Genetic Organisms". These organisms form a comparing standard for all other organisms and a concentrated research on the genetics of them serves as a biological window to all the rest of the species within that phylum (Fink, 1998). The high sequence similarity between many genes from plants and other organisms connects the biological study of plants to all others, and greatly expands the amount of biological knowledge that can be shared between plant biologists and biologists in other fields (Somerville, 2000).

1.2 Biology of *Arabidopsis*

Arabidopsis thaliana is a member of the *Brassica* genus with a broad distribution in nature throughout Europe, Asia, and Northwestern Africa (Meyerowitz and Somerville, 1994). It can complete its whole life cycle within 6 weeks, from seed germination and bolting of the main stem to flowering and seed maturation. Bolting usually occurs about 3 weeks after sowing, during which shoot apical meristem becomes inflorescence meristem and flowers start to be produced. Flowers are small with a length of about 2 mm and self-pollinating. They are composed of four concentric whorls of distinct floral organs, which are sepals, petals, stamens and carpels sequentially from the outermost whorl to the innermost. Genetic crossing can

be easily done by applying pollen of one plant to the stigma surface of another. Plants are usually grown either in small pots filled with soil or in petri dishes placed either under fluorescent lights in the laboratory or in a greenhouse. Healthy mature *Arabidopsis* plants are able to reach a height of 15 to 20 cm and generate several hundred siliques with more than half a thousand seeds in total (Meinke et al., 1998).

1.3 Shoot Apical Meristem (SAM) Organization

During embryogenesis, *Arabidopsis* plants produce apical meristems at both root and shoot ends. The root and shoot apical meristems continuously make new cells throughout the life of the plant to produce the underground root system and the above-ground architecture, respectively. *Arabidopsis* meristems are composed of small groups of pluripotent stem cells that are morphologically undifferentiated (Fletcher, 2002).

The shoot apical meristem (SAM) consists of three radial domains, the central zone, the peripheral zone and the rib zone (Steeves and Sussex, 1989). The central zone comprises a reservoir of stem cells which occupy the apex of the SAM and divide infrequently as compared with other cells in the SAM. Division of the cells in the central zone gradually displaces the progeny cells into the surrounding peripheral zone, where cells divide more often than the

ones at the central zone (Medford et al., 1992; Reddy et al., 2004; Steeves and Sussex, 1989). However, cells in the peripheral zone are more restricted in their differential potency than those at apex and become integrated into either lateral organ or internode primordia (Irish and Sussex, 1992; Steeves and Sussex, 1989). Underneath the central zone and in the deep layers of the meristem lies the rib zone, which forms the pith of SAM and gives rise to the most part of the stem (Steeves and Sussex, 1989). Cell divisions occurring in the rib zone lead to the upward growth of the shoot tips, leaving the cells in the peripheral zone behind to undergo proliferation and differentiation. The peripheral zone is replenished at the same time by descendents of dividing cells from the central zone, which gradually undergo specification with their displacement away from the tip and are essential for the SAM maintenance (Fletcher, 2002).

Another way of dissecting the SAM is to stratify the cells at the apex into distinct layers, named the tunica and corpus (Poethig, 1987; Satina et al., 1940). The tunica is composed of an epidermal L1 layer and a subepidermal L2 layer, each of which is a cell layer of single cell thick and whose cells keep clonally distinct from other cells by dividing solely anticlinally with an orientation perpendicular to the meristem plane (Tilney-Bassett, 1986). The L1 layer cells give rise to the epidermis of leaves, shoots, and flowers, whereas the L2 layer cells are precursors of the germline cells and mesodermal

cells. The corpus, lying beneath the tunica, consists of a group of cells, called L3 cells. The L3 cells produce the vasculature and pith of the stem and innermost cells of lateral organs, such as leaves and flowers. The cell divisions within L3 are orientated more randomly in all planes, differing from those of the L1 and L2 layer cells whose divisions are restricted to a single anticlinal plane (Fletcher, 2002). Although cell divisions are highly organized in the SAM, no fixed patterns exist for SAM cell fate specification based on cell lineage as shown by mosaic analysis (Furner and Pumfrey, 1992; Irish and Sussex, 1992). Since cells that accidentally squeeze from one layer into another layer do not cause defects in development (Tilney-Bassett, 1986), the fate of a SAM cell is decided by its position instead of its clonal origin (Stewart, 1978).

1.4 Stem Cell Maintenance at SAM

The central zone at the tip of the SAM contains stem cell reservoirs that are self-renewal and crucial for the non-stop development and generation of the aerial architectures of higher plants. An intrinsic mechanism of intercellular signaling exists and balances the continuous departure of stem cell derivatives for lateral organ initiation and the constant formation of new stem cell daughters that replenish the stem cell reservoirs (Williams and Fletcher, 2005). Signals that specify stem cell identity are provided by an organizing centre

(OC), which is a small group of *WUSCHEL* (*WUS*) expressing cells beneath the central zone. *WUS*, a homeodomain transcription factor, forms the WOX (*WUS HOMEOBOX*) gene family together with its 14 homologues in *Arabidopsis* (Mayer et al., 1998). *WUS* is both required and sufficient for specifying stem cell identity. Stem cells are mis-specified and SAM is prematurely terminated when *WUS* function is lost (Laux et al., 1996), whereas ectopic stem cell identity is induced when *WUS* is ectopically expressed (Schoof et al., 2000). The neighboring cells above the organizing center are specified to take stem cell identity by the underlying *WUS* activity at the OC. These stem cells express and secrete CLAVATA3 (CLV3) into the extracellular space. CLV3 is a small mobile polypeptide, which binds to the CLV1/CLV2 receptor complex on the membrane of the OC cells and activates the CLV signaling pathway that inhibits *WUS* expression and thereby confines the size of stem cell reservoir (Brand et al., 2000; Lenhard and Laux, 2003; Rojo et al., 2002). This negative feedback loop of regulation between the stem cells and the OC cells maintains the homeostasis of the stem cell population, through an quick adjustment of *WUS* expression following any change in *CLV3* transcription level when the number of stem cells fluctuates (Williams and Fletcher, 2005).

1.5 Major Floral Pathways and Integrators

The shift from vegetative to reproductive growth represents a major transition of development for flowering plants, whose correct timing is crucial for maximizing success of reproduction (Simpson and Dean, 2002). In *Arabidopsis*, flowering time is controlled by multiple genetic floral pathways that have been demonstrated to integrate both endogenous and environmental signals (Fig. 1). The four major pathways are photoperiod pathway, vernalization pathway, autonomous pathway, and gibberellin (GA) pathway (Koornneef et al., 1998; Mouradov et al., 2002; Simpson and Dean, 2002). These genetic pathways respond to different environmental or endogenous signals, but eventually converge to control the expression a set of common targets, which are termed as the floral pathway integrators (Simpson and Dean, 2002). Three genes, which have been identified as the floral pathway integrators, are *LEAFY* (*LFY*), *FLOWERING LOCUS T* (*FT*), and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Kardailsky et al., 1999; Kobayashi et al., 1999; Lee et al., 2000; Samach et al., 2000; Weigel et al., 1992).

The photoperiod pathway responds to changes in day lengths by accelerating flowering under long days. *Arabidopsis* senses light through *CRYPTOCHROME1/2* (*CRY1/2*) and phytochromes A to E (Clack et al., 1994; Lin, 2000), and measures the duration of day or night by an endogenous timer,

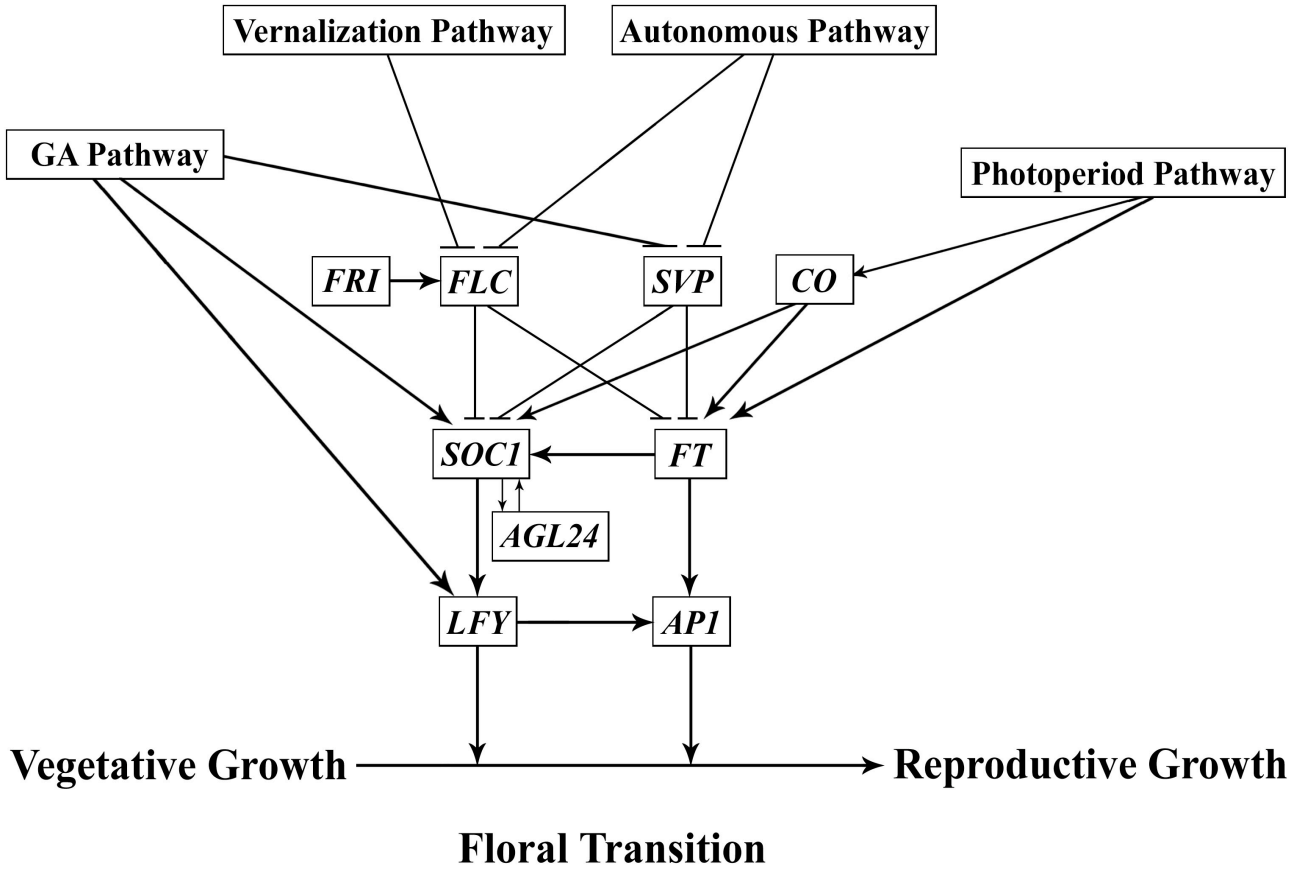

Figure 1. Schematic representation of major genetic flowering pathways and floral pathway integrators. Four major flowering pathways, photoperiod, autonomous, GA, and vernalization, are shown. Floral pathway integrators, *SOC1*, *FT*, and *LFY* integrate flowering signals from several genetic pathways.

called the circadian clock (Thomas and Vince-Prue, 1997). The rhythms of a circadian clock are generated by a central oscillator, which is coupled to regulate physiological activities and adjust its pace according to the light and temperature cycles by multiple pathways (Dunlap, 1999). In *Arabidopsis*, *CONSTANS* (*CO*), a transcription factor with two B-box zinc-finger domains, couples the circadian oscillator to the activation of the flowering-time gene *FT* (Suarez-Lopez et al., 2001). Plants that overexpress *CO* flower early in both short days and long days, whereas loss-of-function *co* mutants are late flowering in long days but not short days (Onouchi et al., 2000). The expression of both *CO* and its target *FT* is altered by mutations that influence circadian rhythms and flowering time (Suarez-Lopez et al., 2001). Under long days, the coincidence between *CO* mRNA expression and CO protein stability allows CO protein accumulation that promotes flowering by inducing expression of three floral integrators, *LFY*, *FT* and *SOC1* (Kardailsky et al., 1999; Kobayashi et al., 1999; Nilsson et al., 1998; Suarez-Lopez et al., 2001). This coincidence is lacking under short day conditions, which explains why *co* mutants flower as wild-type plants in short days (Parcy, 2005).

Vernalization refers to the process that promotes flowering by an extended exposure to cold temperature. Its requirement is adopted by many winter-annual *Arabidopsis* accessions in nature as a reproductive strategy to ensure that they grow vegetatively through the winter and flower until the

favorable spring in the following year (Simpson and Dean, 2002). Dominant alleles at two loci, *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*), are necessary to confer the vernalization requirement in these natural *Arabidopsis* winter-annual accessions (Burn et al., 1993; Clarke and Dean, 1994; Lee et al., 1993). *FLC*, which encodes a MADS-box transcription factor, is a potent repressor of flowering (Michaels and Amasino, 1999; Sheldon et al., 1999). *FRI*, encoding a novel protein with two coiled-coil domains, represses floral transition through its promotive action on *FLC* mRNA abundance (Johanson et al., 2000; Michaels and Amasino, 1999; Michaels and Amasino, 2001; Sheldon et al., 1999; Sheldon et al., 2000). High levels of *FLC* expression repress *FT* expression in leaves and FLC protein also antagonizes meristem response to flowering signals by inhibiting *SOC1* and the *FT* cofactor *FD* expression in meristem (Abe et al., 2005; Corbesier et al., 2007; Searle et al., 2006; Wigge et al., 2005). The vernalization pathway promotes flowering by repressing *FLC* expression and maintaining a repressed state of its chromatin through various epigenetic mechanisms (Bastow et al., 2004; He et al., 2003; Sung and Amasino, 2004).

The autonomous pathway is defined by a group of mutants (*fca*, *fy*, *fpa*, *ld*, *fld*, and *fve*) that are late-flowering independently of photoperiods and highly sensitive to vernalization treatment (Koornneef et al., 1991; Martinez-Zapater and Somerville, 1990; Sanda and Amasino, 1996). Much higher levels of *FLC*

mRNA than wild type have also been shown to be common to this group of mutants, and responsible for their late flowering phenotype that is suppressed in loss-of-function mutants of *FLC* (Michaels and Amasino, 1999; Michaels and Amasino, 2001; Sheldon et al., 1999). Therefore, the autonomous pathway in wild-type plants promotes flowering and converges with the vernalization pathway by negatively regulating the transcription of *FLC* (Mouradov et al., 2002). Although whether an endogenous input signal to the autonomous pathway exists remains unknown, recent studies have shown that flowering control by ambient temperature is mediated by the autonomous pathway in an *FLC*-independent manner (Blazquez et al., 2003).

The gibberellin pathway mediates the effect of GA in promoting flowering. Bioactive GAs are a class of diterpenoid-acid phytohormones that are involved in regulation of diverse aspects of plant development, such as stem elongation, seed germination, and floral induction and development (Yamaguchi, 2008). Exogenous GA application was initially used to demonstrate the promoting effect of GA on flowering (Langridge, 1957), which was substantiated by the study on the GA signaling mutant *gai* that flowers late under both long days and short days even in the presence of GA (Peng et al., 1997), and GA biosynthesis mutant *ga1-3* that flowers late under long days and extremely late or never flowers under short days (Blazquez et al., 1998; Wilson et al., 1992). The complete rescue of the non-flowering phenotype of *ga1-3* under short

days by loss of both *REPRESSOR OF ga1-3* (*RGA*) and *GIBBERELLIN INSENSITIVE* (*GAI*) function (Dill and Sun, 2001) suggests that GA promotes flowering by relieving plants from the restraint conferred by GAI and RGA (Harberd, 2003). It has also been shown that *LFY* was dramatically down-regulated in *ga1* mutants, whose late-flowering phenotype was suppressed by overexpression of *LFY* as well (Blazquez et al., 1998). A *cis*-element in the *LFY* promoter, which is similar to a MYB factor binding site and binds AtMYB33 protein *in vitro* (Gocal et al., 2001), has been found to mediate *LFY* response to GA independently of its induction by photoperiod (Blazquez and Weigel, 2000). Therefore, the GA pathway, which is crucial for promoting flowering mainly under short days, converges with the photoperiod pathway at the level of *LFY* transcription control (Parcy, 2005).

1.6 *SHORT VEGETATIVE PHASE* (*SVP*)

SHORT VEGETATIVE PHASE (*SVP*), which encodes a MICK-type MADS-box transcription factor, is a dosage-dependent repressor of flowering and maintains the duration of the vegetative phase in *Arabidopsis* (Hartmann et al., 2000). The loss-of-function *svp-41* mutants flower much earlier than wild-type plants under both long days and short days, while overexpression of *SVP* driven by CaMV promoter results in extremely late flowering phenotype (Hartmann et al., 2000; Li et al., 2008). SVP has been shown to mediate the

signaling of ambient temperature within the thermosensory pathway by controlling *FT* expression (Lee et al., 2007). A more recent study shows that *SVP* mainly responds to internal signals from GA and autonomous pathways, and the function of SVP as a flowering repressor is mediated by a mutually dependent interaction with FLC protein to form heterodimers that bind to the promoter regions and suppress the transcription of the floral pathway integrators, *SOC1* and *FT* (Li et al., 2008). *SVP* mRNA is expressed throughout the whole seedlings during vegetative phase, but can hardly be detected in the apical meristem of the main inflorescence (Liu et al., 2007). The fact that late flowering phenotype of *ft-1 soc1-2* double mutants is dramatically rescued by the introduction of *svp-41* allele (Li et al., 2008) suggests that there are genes besides *FT* and *SOC1* that are targeted and regulated by SVP in the repression of flowering.

Phylogenic analysis has shown that SVP belongs to the *StMADS11*-like clade of MADS-box proteins (Fig. 2) that consists of members from gymnosperms, monocts, and eudicots (Becker and Theissen, 2003). The expression of the majority of its members is localized to vegetative tissues, and several members have been reported as flowering repressors (Hartmann et al., 2000; Kane et al., 2005; Masiero et al., 2004).

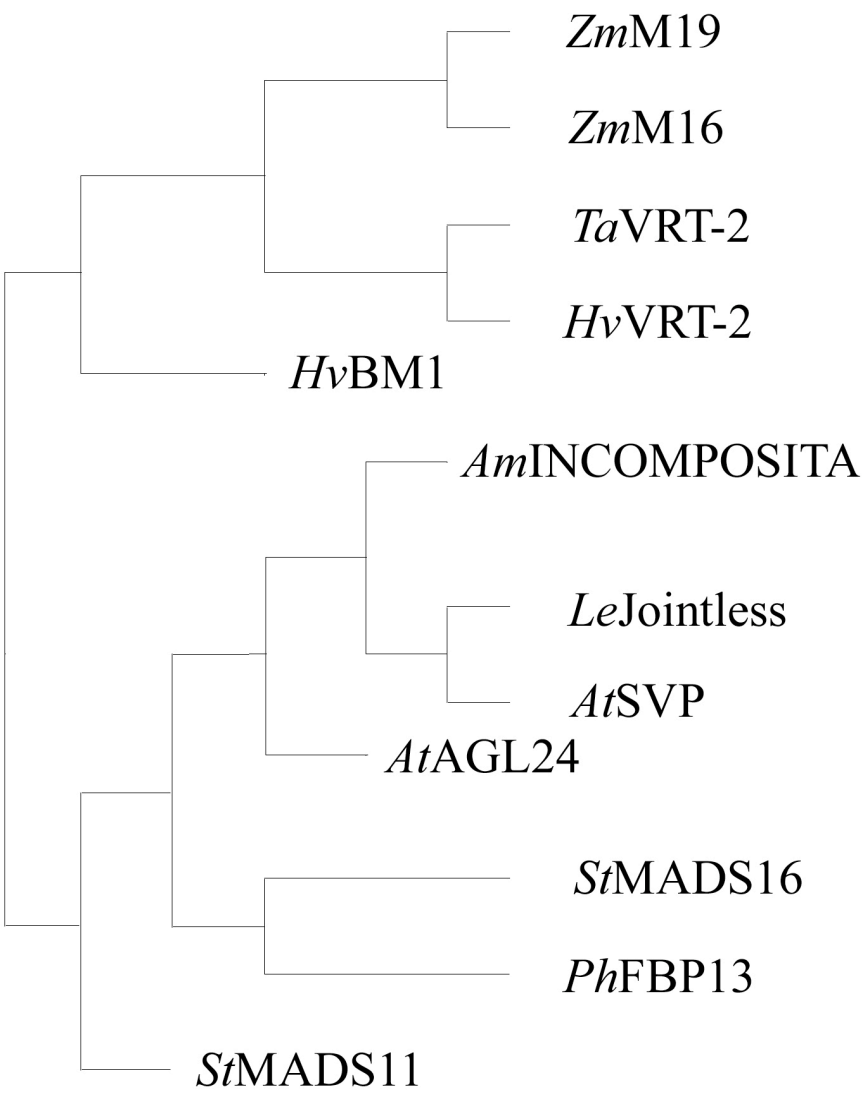

Figure 2. Phylogenetic tree of StMADS11 clade. SVP belongs to the StMADS11-like clade of MADS-box gene family. Major MADS-box regulatory proteins of this subfamily in monocots and dicots are illustrated within this phylogenetic tree (Becker and Theissen, 2003).

1.7 MADS-Box Gene Family

MADS-box genes encode a group of transcription factors that play fundamental roles in diverse biological processes in almost all eukaryotes (Riechmann and Meyerowitz, 1997; Shore and Sharrocks, 1995). The MADS-box is a highly conserved DNA sequence of about 180 bp in length that encodes a DNA-binding domain with dimerization and accessory factor binding functions of all the family members, and is named after the initials of its four founder proteins, MCM1 (from *Saccharomyces cerevisiae*), AGAMOUS (from *Arabidopsis*), DEFICIENS (from *Antirrhinum*), and SRF (from *Homo sapiens*) (Schwarz-Sommer et al., 1990; Shore and Sharrocks, 1995). In accordance with their conserved DNA-binding domains, MADS-box transcription factors bind to similar DNA sequences with a consensus motif $CC(A/T)_6GG$, called CArG box, that are commonly present in the promoter regions of genes controlled by MADS-box proteins (Shore and Sharrocks, 1995; Tilly et al., 1998). MAD-box genes from plants have been categorized into three types, termed as type I, type II, and MADS-like genes (De Bodt et al., 2003). While the function of type I MADS-box genes from plants remains almost entirely unknown (Alvarez-Buylla et al., 2000; De Bodt et al., 2003), the plant type II genes are much better understood due to the fact that all the well-characterized MADS-box genes with known mutant phenotypes or detailed expression patterns belong to this category (Becker and Theissen,

2003). Plant type II genes are also called MIKC-type genes, as they all share a conserved organization of structure, that includes a MADS (M-), intervening (I-), keratin-like (K-) and C-terminal (C-) domain (Ma et al., 1991; Munster et al., 1997; Theissen et al., 1996). The I-domain is less conserved and determines selectivity for DNA-binding dimer formation (Riechmann and Meyerowitz, 1997), while the K-domain consists of conserved hydrophobic residues that are regularly spaced and presumably involved in dimerization by forming an amphipathic helix (Ma et al., 1991; Shore and Sharrocks, 1995). The C-domain, which is the most variable in both sequence and size, participates in activating transcription or forming multimeric transcription factor complexes (Cho et al., 1999; Egea-Cortines et al., 1999).

1.8 *Arabidopsis Protein Interacting with NIMA-1* (*AtPIN1*)

AtPIN1 encodes a 119 amino-acids protein with a molecular mass of 13kDa and was identified as the first PIN1-type parvulin from *Arabidopsis* (He et al., 2004; Landrieu et al., 2000). Multiple sequence alignment showed that non-plant PIN1 homologs contain two domains, a regulatory WW domain and a catalytic PPIase domain, whereas *Arabidopsis* PIN1 possesses only a single PPIase domain (Landrieu et al., 2000).

PIN1-type peptidyl-prolyl *cis/trans* isomerases include members like Protein

Interacting with NIMA-1 (PIN1) from human (Lu et al., 1996), and Essential (ESS1)/Processing/Termination Factor 1 (PTF1) from budding yeast (Hanes et al., 1989; Hani et al., 1995). It has been proposed that these proteins may function as novel molecular timers that regulate the amplitude and duration of diverse cellular responses or processes, such as neuronal function, responses to growth signaling and cellular stress, progression of cell cycle, and immune responses (Lu and Zhou, 2007). These enzymes, as well as AtPIN1, recognize only phosphorylated Ser/Thr residues preceding proline (pSer/Thr-Pro) that normally takes one of two distinct confirmations: *cis* and *trans* (Hani et al., 1999; Landrieu et al., 2000; Yaffe et al., 1997). By interacting with phosphorylated substrates as described, PIN1 homologs are able to catalyze their conformational changes and thereby regulate their biological functions (Liou et al., 2003). Due to the fact that phosphorylation of Ser/Thr-Pro is adopted by organisms as a key regulatory mechanism to control various cellular processes, the PIN1-catalyzed prolyl isomerization represents an important post-transcriptional and post-phosphorylation regulatory mechanism.

1.9 *Kip-Related Proteins* (*KRP*s)

In mammals, PIN1 regulates the transcription of the cell cycle arrest genes, $p21^{Cip1}$ and $p27^{Kip1}$ (Brenkman et al., 2008; Wulf et al., 2002), which belong to

the Cip/Kip family. As one of the only two subfamilies of cyclin-dependent kinase inhibitors (CKIs) (Pavletich, 1999), Cip/Kip family was shown to control the G1/S and G2/M transitions by forming protein complexes with different cyclin/CDK complexes (Nakayama and Nakayama, 1998).

In *Arabidopsis*, Kip-related proteins 1 – 7 (KRP1 – KRP7) have been identified with sequence similarity to $p27^{Kip1}$ (De Veylder et al., 2001; Lui et al., 2000; Wang et al., 1997; Zhou et al., 2002). Studies have revealed that these *KRP* genes were differentially expressed in *Arabidopsis* plants, with *KRP1* and *KRP2* restricted to endoreduplicating cells, *KRP4* and *KRP5* to mitotically dividing tissues, and *KRP3*, *KRP6* and *KRP7* in both endoreduplicating and mitotically dividing regions (Ormenese et al., 2004). Recent studies have shown that KRP1 is involved in the G1/S transition of the cell cycle by interacting with CDKA;1/CYCD2;1 complex in *Arabidopsis* and may be targeted by ubiquitin / proteasome pathway mediated by SCF^{SKP2} and a RING protein RKP (Ren et al., 2008). Misexpression of *KRP1* in *Arabidopsis* trichomes has been reported to show diminished endoreduplication and cell size, and induced apoptosis (Schnittger et al., 2003).

1.10 Conclusion

Since the adoption of *Arabidopsis thaliana* as a model organism for plant research and the availability of modern molecular tools, our understanding of various aspects of plant development has been enormously improved, with the ABC model proposed to explain flower development, multiple flowering pathways molecularly characterized, and hormones and florigen demystified, etc. Although many more questions remain to be answered, the prospect is still bright in view of the unparalleled power of biology unleashed by modern technology and molecular tools that are available or to be made available. Flowering represents one of the most complex and dynamic processes, which is regulated at multiple levels and coordinated by multiple pathways to ensure that reproduction success is achieved under a variety of conditions. Since many fundamental mechanisms are conserved broadly, research on flowering not only help plant biologists, but also provides insights into research in other fields. Beyond the attraction to understand the flowering process out of scientific curiosity, the relevant knowledge holds keys to many problems in daily life, from increasing yields of crops, maintaining fruits in good shape, to producing novel decorative flowers.

SVP encodes a MIKC-type MADS-box transcription factor. Its loss-of-function *svp-41* mutants are very early flowering under both long days and short days, while constitutive expression of *SVP* under 35S promoter leads

to extremely late flowering (Hartmann et al., 2000; Li et al., 2008). It has been shown in a recent study that *SVP* mainly responds to internal signals from GA and autonomous pathways, and its role as flowering repressor is mediated by a mutually dependent interaction with FLC protein by forming heterodimers that associate with the promoter regions and suppress the transcription of the floral pathway integrators, *SOC1* and *FT* (Li et al., 2008). *SVP* mRNA is expressed throughout the whole seedlings during vegetative phase, but is hardly detectable in the apical meristem of the main inflorescence (Liu et al., 2007). The fact that late flowering phenotype of *ft-1 soc1-2* double mutants is dramatically rescued by the introduction of *svp-41* allele (Li et al., 2008) indicates that other target genes of SVP exist, besides *FT* and *SOC1*, in control of flowering. Therefore, in this study, we investigated target genes of SVP in the regulation of flowering time and performed functional characterization of identified target genes.

Chapter 2 Materials and Methods

2.1 Plant Materials and Growth Conditions

All mutants of *Arabidopsis* used in this study are in the Columbia (Col) background unless otherwise claimed. To break dormancy, all seeds were sown and placed under 4°C for 3 days before moved to growth rooms. All the plants were grown at 22°C under short days (8 hr light/16 hr dark) or long days (16 hr light/8 hr dark). *svp-41* mutant was provided by Peter Huijser (Max-Planck Institute, Germany) and SALK line insertion mutants were purchased from the *Arabidopsis* Biological Resource Center (Ohio State University, USA). The transgenic lines in study were made by transforming each construct into wild-type Col plants using *Agrobacterium*-mediated floral dipping method and screened with 3% BASTA after the emergence of the first rosette leaf.

2.2 RNA Extraction

Total RNA was extracted using RNeasy® Plant Mini Kit (QIAGEN, USA) according to the manufacturer's instructions. All pipette tips and Eppendorf tubes were autoclaved at 121°C for 1 hr before use. About 100 mg of aerial parts or leaves or SAM from plants were frozen and grounded thoroughly in

liquid nitrogen using a pestle and a mortar. The sample was then moved to a 1.5 ml Eppendorf tube with 450 µl Buffer RLT and vortexed vigorously. The lysate was transferred directly into a QIAshedder spin column sitting in a 2 ml collection tube by pipetting. After 2 min of centrifugation at maximal speed, supernatant of the flow-through fraction was carefully pipetted to a new Eppendorf tube without disturbing the cell-debris pellet at the bottom of the collection tube. For each volume of the clear supernatant, 0.5 volume 100% ethanol was added and mixed immediately by pipetting or vortexing. The well mixed sample was then pipetted to a RNeasy® mini column placed in a new 2 ml collection tube. After 30 s of centrifugation at maximal speed, the flow-through was discarded and 700 µl of Buffer RW1 was applied to the RNeasy® mini column, before washing the column and centrifugating for another 30 s at maximal speed. The RNeasy® mini column was then placed into a new 2 ml collection tube after discarding the collection tube with the flow-through. Subsequently, the RNeasy® mini column was added with 500 µl of Buffer RPE and then centrifuged for 30 s at top speed. The washing of the RNeasy® mini column with Buffer RPE was repeated once more, before the RNeasy® mini column was moved to a new 1.5 ml Eppendorf tube. 100 µl of RNAse-free water was used for RNA elution by directly pipetting onto the silica-gel membrane of the RNeasy® mini column. Elution efficiency could be further increased by repeating the elution step with the first eluate. DNA contaminations could be removed from the total RNA samples, by incubating

total RNA extracts on the RNeasy® mini column with RNAse-free DNAse (QIAGEN, USA) at 37°C for 30 min between the two washing steps before the final elution.

2.3 Reverse Transcription for cDNA Synthesis

Synthesis of cDNA was performed by reverse transcription reaction using ThermoScript™ II RT-PCR system (Invitrogen, USA) according to the manufacturer's instructions. Each reaction system was assembled with 0.5 µl of 50µM Oligo$(dT)_{20}$, 5 pg to 2.5µg of total RNA, and 1 µl of 10 nM dNTP Mix, in a PCR tube and adjusted to 6 µl in volume. The total RNA and primer were then denatured by incubating at 65°C for 5 min and placed on ice immediately. To each reaction tube placed on ice, 2 µl of 5X cDNA Synthesis Buffer, 0.5 µl of 0.1 M DTT, 0.5 µl of 40 U/µl RNaseOUT™, 0.5 µl of DEPC-treated water, and 0.5 µl of 15 units/µl ThermoScript™ RT were added and mixed well by pipetting. The 5X cDNA Synthesis Buffer needed to be vortexed for 5 s right before use. The reaction tubes were subsequently moved directly from ice to a thermal cycler preheated to 50°C, and incubated for 30 to 60 min at 50°C. The reaction was terminated by incubation at 85°C for 5 min and added with 1 µl of DNase-free RNase H for incubation of another 20 min to remove the RNA templates. The synthesized cDNA reactions were stored at -20°C or used for real-time or semi-quantitative PCR immediately.

2.4 Expression Analysis

2.4.1 Quantitative Real-time PCR

In order to quantify the mRNA level of target genes and compare the expression difference of these genes between different genotypes, triplicates of quantitative real-time PCR on diluted aliquots of reverse-transcribed cDNA templates were performed with SYBR Green PCR Master Mix (Applied Biosystems, USA) on 7900HT Fast Real-Time PCR system (Applied Biosystems, USA) using *TUBULIN2* (*TUB2*) as an endogenous control. The cycle threshold (Ct) difference between the target gene and the control *TUB2* ($\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{tubulin}}$) was used for computation of the normalized expression levels of target genes, which are equal to $2^{-\Delta Ct}$. The specificity of real-time primers was evaluated by examining the plot of dissociation curve for any abnormal amplification or bimodal dissociation curve, while the efficiency were determined by plotting a standard curve base on a series of 10-fold dilutions of DNA templates for each pair of primers.

2.4.2 Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed by PCR amplification, using specially designed primers, on diluted aliquots of reverse-transcribed cDNA

templates. The amplified PCR products were either fractioned on an agarose gel directly or followed by hybridization with labeled probes. PCR primers to semi-quantify gene expression were designed using the on-line software Primer3 available at http://frodo.wi.mit.edu (Rozen and Skaletsky, 2000). Following criteria were used to choose primers: length between 18 and 22 bp, T_m between 58°C and 62°C, and length of amplification product between 400 and 600 bp. Expression level of *TUBULIN2* (*TUB2*) was used as internal control for normalization purpose.

2.5 Non-radioactive *in situ* Hybridization

Non-radioactive *in situ* hybridization was conducted as previously described (Yu et al., 2004).

2.5.1 RNA Probe Synthesis

Although either DNA or RNA probes can be used for *in situ* hybridization, RNA probes give better sensitivity and stronger signals. Therefore, RNA probes were used for all *in situ* hybridization experiments in our study. Genes were cloned into pGEM-T Easy vector (Promega, USA) as an insert, which is flanked by SP6 and T7 promoters on each side, respectively. Before either SP6 or T7 polymerase was used to generate mRNA transcripts, the plasmids were

linerized by digestion with appropriate and sufficient restriction enzyme that leaves no 3' overhang and ensures complete cutting. Phenol/chloroform extraction was performed twice followed by precipitation to remove any RNases. Digested plasmids were then resuspended at 0.5 µg/µl in DEPC-treated water. A Digoxigenin (DIG) RNA Labeling Kit (Roche, Germany) was used for labeling RNA probes to be generated from digested plasmids. Transcription and labeling reaction was set up by mixing 1 µg of linerized plasmids, 2 µl of 10x DIG labeling Mix, 1 µl of RNase inhibitor (Promega, USA), 2 µl of RNA polymerase (Promega, USA), and RNase-free H_2O to a total volume of 20 µl. Incubation of the reaction was kept at 37°C for 2 hr, before the addition of 2 µl of RNase-free DNase (Roche, Germany) and incubation at 37°C for another 30 min to get rid of DNA templates. The success of the reaction could be checked by running 1 µl of the products on an agarose gel for about 15 min. The DIG labeled RNA probes were then cut into pieces between 75 and 150 bp in length by carbonate hydrolysis to increase tissue permeability. Calculation of reaction time for alkaline treatment was conducted using the following series of formula: Time = $(L_i - L_f) / 0.11 * L_i * L_f$; L_i = initial length of probe (in kb); L_f = final length of probe (0.15 kb was used for calculation). Hydrolysis of the labeled RNA probes were performed by mixing the transcription reaction, filled to 100 µl with DEPC water, with 100 µl of 2xCO_3 buffer (80 mM $NaHCO_3$, 120 mM Na_2CO_3) and incubating at 60°C for a period of the calculated time. The reaction was subsequently

neutralized with 10 µl of 10% acetic acid, and then precipitated at -20°C from 1 hr to overnight, with a mixture of 1/10 volume of 3M NaAc (pH 5.2), 2.5 volumes ethanol, and 2 µl of 10 mg/ml tRNA. The pellet obtained was washed with 70% EtOH and resuspended in 80 µl of 50% formamide. The probes were used at a final concentration of 0.5 ng/µl/kb in the hybridization solution. Optimal concentration was determined by trying up to 5x higher or lower of the forementioned concentration.

2.5.2 Material Fixation

The fixative for the *in situ* hybridization contains 4% (weight / volume) of paraformaldehyde dissolved in 1x PBS solution (0.13 M NaCl, 7 mM Na_2HPO_4, and 3 mM NaH_2PO_4, pH 7.0). The fixative was prepared by firstly adjusting the pH value of required amount of 1x PBS solution to 11 and then heating to 60-70°C in a water bath or on a heat block. Appropriate amount of paraformaldehyde was added and dissolved in the PBS solution in a fume hood by vigorous shaking, which was then chilled on ice. When the PBS solution was cooled to 4°C, H_2SO_4 was used to adjust its pH value to 7 for immediate use of plant material fixation. Plant tissues were collected into ice-cold fixative solution, and then vacuum was applied until bubbles started to form in the fixative. The vacuum was held for 15 min and then released slowly, which was repeated until samples began to sink. The fixative was

subsequently replaced with new fixative, and gentle shaking continued overnight at 4°C.

2.5.3 Dehydration and Embedding

After samples were fixed with paraformaldehyde, they were washed twice with 1x PBS solution at 4°C for 30 min each. This was followed by dehydration through a series of ethanol washing with ethanol concentrations of 30%, 40%, 50%, 60%, 70%, and 85%, for 60 min at 4°C each time with shaking. The samples were then washed at 4°C overnight with shaking by 95% ethanol supplemented with eosin which stained plant tissues for visualization of samples later in wax. Further dehydration with 100% ethanol plus eosin continued under room temperature for four times with the first two lasting 30 min each and the last two 60 min each. The samples were subsequently infiltrated with histoclear by a series of washing with ethanol solutions of increasing concentration of histoclear. Histoclear infiltration of the samples started with immersion of the samples in 25% histoclear and 75% ethanol at room temperature for 60 min and was followed by sequentially washing for 60 min each at room temperature on a rocker with 50% histoclear and 50% ethanol, and 75% histoclear and 25% ethanol. The samples were immerged with 100% histoclear at room temperature on a rocker for another three times, with the first two times lasting 60 min each and the third one

overnight with 1/4 volume of paraplast chips added. On the next day, the samples were placed at 42°C until the paraplast chips melted completely and another 1/4 volume of chips were added. After the newly added chips melted completely, the materials were moved to 55°C for several hours and then freshly melted chips were used to replace the mixture of the wax and histoclear. The samples were kept at 55°C overnight and on the next day transferred to new freshly-melted wax at 55°C for two times separated by several hours. This continued for another day with two times of wax change per day. On the following day, the plant tissues were embedded in wax by pouring the samples together with wax into a weighing dish that served as a mold at 55°C. The samples were oriented properly with the help of a pre-warmed syringe needle. Bubbles were carefully avoided to be trapped in the wax. The weighing dish was then moved to room temperature for cooling of the paraffin and the solidified wax was stored at 4°C.

2.5.4 Sectioning

ProbeOn Plus slides (Fisher Biotechnology, USA), which were pre-cleaned and charged, were used in our experiments. A white frosting featured on these slides also allowed us to sandwich them in pairs later during hybridization and detection. During sectioning, slides were placed on a RNase-free slide warmer, which was pre-warmed to 42°C. A few drops of DEPC-treated water were

applied onto the slides. Tissue sections were made with a thickness of 8 μm each and ribbons of tissue were floated on top of water on the slides with the shiny side down with the help of a paintbrush or a toothpick. The water was drained off using a Kimwipe, after the ribbons flattened out by sitting for a few minutes. The slides were then incubated on the slide warmer overnight to allow adherence of tissue onto the slides. Slides of sectioned tissue were kept at 4°C under dry conditions for up to several weeks.

2.5.5 Pre-treatment of *in situ* Sections

All solutions in this step were made RNase-free, plastic containers were treated with 0.1 M NaOH overnight and rinsed with sterile water and all glassware and stirbars were autoclaved at 121°C for 1 hr before use.

Deparaffinization was performed on a low-speed rocker at room temperature. It started with two times of washing with 100% histoclear each for 10 min, followed by two times of washing with 100% ethanol for 1-2 min each time. Washing with ethanol solution continued with ethanol concentrations of 95%, 90%, 80%, 60%, and 30%, sequentially, each for 1-2 min. Slides were then washed by H_2O for 1-2 min and 2x SSC (0.3 M NaCl, 30 mM Na Citrate) for another 15-20 min. Prewarmed Tris / EDTA solution (100 mM Tris pH 8, 50 mM EDTA) with freshly added proteinase K (1 μg/ml) was used to treat the

slides at 37°C for 30 min, before they were washed by 2 mg/ml glycine in PBS at room temperature for 2 min. The slides were then washed again by PBS solution at room temperature twice for 2 min each time, and incubated in freshly made 4% (weight / volume) paraformaldehyde in PBS solution (pH 7) at room temperature. Additional washing with PBS solutions were repeated twice for 5 min each time at room temperature, followed by a 10 min incubation in 0.1 M triethanolamine (pH 8) and acetic anhydride. The slides were then washed by two times of PBS solution for 5 min each time, and dehydrated through sequential washing with 30%, 60%, 80%, 90%, 95% and 100% ethanol for 30 s each time. The last 100% ethanol washing step was repeated once more, after which the slides were kept at 4°C up to several hours in a container filled with some ethanol at the bottom before used for *in situ* hybridization.

2.5.6 *In Situ* Hybridization

Slides were air-dried completely on clean Kimwipes or paper towels. Every volume of 800 µl of hybridization solution was prepared by mixing 100 µl of 10x *in situ* salts, 400 µl of deionized formamide, 200 µl of 50% dextran sulfate, 20 µl of 50x Denhardts solution (warmed up before pipetting), 10 µl of tRNA (10 mg/ml), and 70 µl of DEPC-treated H_2O. For each pair of slides, a volume of 60 µl of 50% formamide with RNA probes (2.5 ng/µl/kb) was

heated to 80°C for 2 min, cooled on ice, spun down and kept on ice. Every 60 µl of RNA probe solution was mixed with 240 µl of hybridization solution without causing bubbles to give a total volume of 300 µl, all of which was applied to each slide pair by pipetting to the middle of one slide and slowly sandwiching the other slide on top until they were completely together without trapping bubbles. The slides were then elevated above wet paper towels or simply a layer of water in a plastic container, and hybridization was performed by incubation at 50-55°C overnight.

2.5.7 *In Situ* Post-hybridization

After hybridization, slides were washed with a series of different solutions to increase signal noise ratio by reducing background noise. Sandwiched slide pairs after hybridization were separated and rinsed by dipping into 0.2x SSC solution that was prewarmed to 55°C. They were further washed with gentle rocking twice with prewarmed 0.2x SSC for 60 min each time, followed by 5 min washing with PBS at room temperature. Blocking was then conducted by washing the slides for 45 min at room temperature on a rocker with 1% freshly made Boehringer block in 100 mM Tris pH7.5, 150 mM NaCl. The block solution was subsequently replaced and incubated with 1.0% Bovine Serum Albumin (BSA) in 100 mM Tris pH 7.5, 150 mM NaCl, 0.3% Triton X-100, on the rocker for another 45 min at room temperature. Anti-DIG antibody

(Roche, Germany) was diluted to a ratio of 1:500 in the forementioned BSA/Tris/NaCl/Triton solution to make a puddle in a plastic weighing dish. Slides were then sandwiched together, and dipped into the antibody puddle to pull up solution by capillary action. Kimwipe was used to drain the slides and the last step of dipping into the antibody solution was repeated. Great care was taken to avoid bubbles throughout the forementioned process of pulling up antibody solution. Slides were then elevated above a layer of water or wet paper towels in a plastic container and allowed to sit at room temperature for 2 hours, before they were drained on Kimwipes and separated. They were washed for four times with the forementioned BSA/Tris/NaCl/Triton solution for 15 min each time at room temperature on a rocker, followed by washing with 100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$ for 10 min. Each slide was then dipped again in fresh Tris pH 9.5/NaCl/$MgCl_2$ solution to ensure the removal of all detergent. For preparation of substrate solution, a Tris-NaCl-PVA stock solution was first prepared with 10% (weight/volume) polyvinyl alcohol (PVA, either 40 kD or 70-100 kD, Sigma, USA) dissolved in Tris pH 9.5/NaCl/$MgCl_2$ solution. The stock solution was then heat-shocked using microwave oven for a few times, mixed vigorously, chilled at room temperature, and stored at 4°C for up to several months. Substrate solution that was sufficient for 30-50 slides was obtained by adding 200 µl of NBT/BCIP stock solution (Roche, Germany) to 10 ml of Tris-NaCl-PVA stock solution and mixing vigorously. The substrate solution was then placed

in darkness for a while until bubbles were removed. Each slide was covered with at least 150 µl of the substrate solution, sandwiched onto each other in pair, and kept elevated in a plastic container above wet paper towels in the dark for 12-24 hours. Slides were then drained with Kimwipes, and rinsed in tap water for at least three times to terminate reaction. This was followed by dehydration by brief washing with 70% ethanol once and 100% ethanol twice, each for about 10 s. Slides were air-dried at room temperature and mounted with 50% glycerol for observation under microscopes and maintenance of signals for at least three months.

2.6 Chromatin Immunoprecipitation (ChIP) Assays

Seven-day-old seedlings of *svp-41 SVP:SVP-6HA* were fixed at 4°C for 40 min in 1% formaldehyde under vacuum, followed by homogenization of the fixed tissues. Chromatin was then extracted and sonicated to produce DNA fragments below 500 bp. The solubilized chromatin was placed under incubation with anti-HA agarose beads (Sigma, USA) for 90 min at 4°C after sparing a portion to be used as input control. Washing of the beads were performed five times, followed by incubation of the beads with the elution buffer supplemented with 1 µl RNase A (1 mg/ml) for 30 min at 37°C. The coimmunoprecipitated DNAs were then recovered and tested for enrichment by quantitative real-time PCR analysis. ChIP assays were performed in

replications of at least three independent rounds, and real-time PCR assays were performed in triplicates. Calculation of the relative enrichment of each fragment was completed by first normalizing the amount values of all DNA fragments against the value of a genomic fragment of *ACTIN* as an endogenous control, and then by normalizing the value of transgenic plants against that of the wild-type plants as a negative control using the equation $2^{(\text{CtSVP-6HA Input} - \text{CtSVP-6HA ChIP})}/2^{(\text{CtWT Input} - \text{CtWT ChIP})}$.

2.7 Microarray Experiments

For comparison of transcriptomes between *svp-41* mutants and wild-types plants, three sets of samples that are biologically independent were used for our study. An amount of 5 μg of total RNA extracted from each set of replicates was used to synthesize double-stranded cDNA, followed by *in vitro* transcription to obtain labelled complimentary RNA (cRNA) using the One-Cycle Target Labeling and Control Reagents Kit (Affymetrix, USA) according to the manufacturer's protocols. After subsequent cleaning up and fragmentation, hybridization of the cRNAs was performed on the Affymetrix Arabidopsis ATH1 high-density genome arrays for 16 hr. After hybridization, the arrays were washed and stained using GeneChip Fluidics Station 450. Scanning of the probe arrays was made by GeneChip Scanner, and signals were captured by GeneChip Operating Software (GCOS). GeneSpring GX

7.3.1 (Agilent) was used to analyze the .CHP files generated by GCOS. Normalization of all samples was made to the 50th percentile for each chip and to median signals for each gene, and the data interpretation incorporated cross-gene error model. Filtering of genes by Affymetrix flags was used to reduce number of genes. Definitions of genes that were either up- or down-regulated were made independently as changes of gene expression levels that were statistically significant in comparison between *svp-41* mutants and wild-type controls.

2.8 Genomic DNA Extraction

2.8.1 Rapid Extraction of Genomic DNA

In a 1.5 ml Eppendorf tube, plant leaves were submerged and ground in 200 µl of extraction buffer, which consists of 0.2 M Tris-HCl pH 9.0, 25 mM EDTA, 1% SDS, and 0.4 M LiCl. The tissue suspension was then centrifuged at maximal speed for 5 min, before the supernatant was moved to a new 1.5 ml Eppendorf tube and mixed well with 150 µl of isopropanol. After centrifugation at maximal speed for 10 min, the supernatant was discarded and the cell pellet was washed by adding 500 µl of 70% ethanol. The pellet was resuspended and centrifuged for 5 min at maximal speed. Again, the supernatant was discarded and the cell pellet was either air-dried by placing the tube upside down on a paper towel or dried by vacuum. The extracted

genomic DNA was then resuspended in 100 µl of either sterile water or TE buffer (10 mM Tris pH 8.0, 1 mM EDTA), and stored at -20°C.

2.8.2 Kit-facilitated Extraction of Genomic DNA

For extraction of genomic DNA with improved quality, the DNeasy Plant Mini Kit (Qiagen, USA) was used. Up to 100 mg (wet weight) of plant samples were disrupted either after freezing in liquid nitrogen without Buffer AP1 or directly in Buffer AP1 without using liquid nitrogen. The former disruption method needed to have 400 µl of Buffer AP1 and 4 µl of RNase A stock solution added immediately to the sample after disruption, and would yield DNA of a higher molecular weight, while the latter one yielded DNA ideal for PCR but might shear DNA of higher molecular weight. The disruption itself was performed either using a mortar and a pestle or a Tissue Lyser machine. The sample was then vortexed vigorously and incubated for 10 min at 65°C, during which the tube was inverted 2 or 3 times to keep the sample mixed. Immediately after incubation, 130 µl of Buffer AP2 was added and mixed with the lysate, and the mixture was incubated on ice for 5 min, before it was centrifuged for 5 min at 14,000 rpm. The supernatant was then applied to a QIAshredder Mini spin column sitting in a 2 ml collection tube, and centrifuged for another 2 min at 14,000 rpm. The flow-through fraction was then carefully moved into a new 1.5 ml Eppendorf tube without causing

disturbance to the pellet of cell debris. 1.5 volumes of ethanol-added Buffer AP3/E were then pipeptted to the clear lysate and mixed immediately. The mixture, including any precipitate, was subsequently transferred into a DNeasy Mini spin column resting in a 2 ml collection tube, and centrifuged for 1 min at 8000 rpm or higher. The flow-through was discarded and the DNeasy Mini spin column was then placed into a new 2 ml collection tube. 500 µl of ethanol-included Buffer AW was then added into the DNeasy Mini spin column and centrifuged for 1 min at 8000 rpm at least. With the flow-through discarded and the collection tube reused, another 500 µl of ethanol-added Buffer AW was pipetted to the DNeasy Mini spin column and centrifuged for 2 min at 8000 rpm or more to let dry of the membrane. Following the centrifugation, the DNeasy Mini spin column was removed from the collection tube carefully without any contact with the flow-through, and placed into a new 1.5 ml or 2.0 ml Eppendorf tube. Elution of DNA from the DNeasy Mini spin column was achieved by applying 100 µl sterile water or Buffer AE directly onto the DNeasy membrane and incubating for 5 min at room temperature, before centrifuging for 1 min at 8000 rpm or more. The elution step could be repeated once more with or without a new Eppendorf tube. The genomic DNA extracted thereby was then either used directly for PCR or stored at -20°C.

2.9 Competent Cell Preparation

Competent cells of *Escherichia coli* for heat-shock transformation were prepared using a protocol adapted from previously described (Inoue et al., 1990). The XL1-Blue strain of *E. coli* (Stratagene, USA) was chosen and used for routine cloning in the lab. This strain is both endonuclease (*endA*) and recombination (*recA*) deficient, dramatically improving the quality of miniprep DNA and insert stability, respectively. Frozen stock of XL1-Blue *E. coli* cells from -80°C was thawed on ice, streaked onto a LB agar plate, and cultured overnight at 37°C. A single colony was picked and inoculated into 1.5 ml of SOB medium (yeast extract 5 g/l, Tryptone 20 g/l, NaCl 0.58 g/l, KCl 0.19 g/l, $MgCl_2 \cdot 6H_2O$ 2.03 g/l, $MgSO_4 \cdot 7H_2O$ 2.46 g/l) in a 15 ml culture tube and incubated for 12 hr by vigorously shaking (200 rpm) at 37°C. An aliquot of 500 µl of the overnight culture was transferred to 100 ml fresh SOB medium in a 1-liter flask and cultured to an A_{600} value of 0.6 at 20°C in a vigorously shaking incubator (225 rpm). The culture was subsequently loaded into two ice-cold Falcon tubes and cooled on ice for another 10 min before centrifuging at 3000 rpm for 5 min at 4°C. After the supernatant was discarded, the pellet at the bottom of each Falcon tube was gently resuspended in 20 ml of TB medium freshly prepared (10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7), placed on ice for 10 min, and centrifuged for 5 min at 4 °C. After discarding the supernatant, the cell pellet at the bottom of each tube was gently re-suspended again in 4 ml of fresh ice-cold TB medium. A final

concentration of 7% of DMSO was added with gentle swirling to the cell suspension as a stabilizer. To each pre-chilled 1.5 ml Eppendorf tube, an aliquot of single-use amount of the cell suspension was immediately pipetted. These tubes of cell suspension were then quick-frozen by immersion into liquid nitrogen, and kept at -80°C for long term storage up to several months without dramatic decrease of competency.

2.10 Transformation of *E. coli* Competent Cells

2.10.1 Heat Shock

Each tube with an aliquot of 50 µl of frozen competent cells was placed on ice and thawed. An amount of 2 µl of ligation reaction was pipetted to each tube and mixed sufficiently with the competent cells by gentle tapping of the tube. After incubation on ice for 30 min, the mixture was then placed in a water bath or a heat block preheated to 42°C and heat-shocked at 42°C for 90 s. The tubes were then placed on ice for an incubation of another 2 min, before adding 1 ml LB medium to each tube and culturing at 37°C for 1-2 hr with shaking at 250 rpm. Each culture was subsequently centrifuged at room temperature for 7 min at 6000 rpm. The supernatant was discarded, and the cell pellet was resuspended by water. The cell suspension was then spread evenly onto a LB agar plate, which contains specific antibiotics that are required for plasmid selection. The LB agar plates were then incubated

overnight at 37°C. For pGreen vectors, LB agar plates were supplemented with 50 mg/ml kanamycin, while for pGEM-T easy vector, 100 mg/ml of ampicillin, 20 µl of 2% X-gal, and 100 µl of 100 mM IPTG were added into the LB agar plates for blue/white colony screening.

2.10.2 Verification of Constructs by Colony PCR

Colonies on the surface of the LB agar medium were picked up by autoclaved pipette tips and then resuspended in 5 µl of sterile water in 0.2 ml Eppendorf tubes. An amount of 2 µl of the cell suspension was used as DNA template for PCR reaction. A gene-specific primer and a vector specific primer were used for amplification, and the PCR products were then separated by electrophoresis on 1% agarose gels. Colonies that generate DNA fragments of expected sizes were inoculated in 3 ml of LB medium and cultured for 24 hr at 37°C.

2.10.3 Plasmid DNA Extraction

Plasmid DNA was extracted with the High-Speed Plasmid Minikit (Geneaid, Taiwan) according to manufacturer's protocol. Cultured cells were transferred to a 1.5 ml Eppendorf tube and harvested by centrifugation for 1 min at maximal speed. The supernatant was discarded and the harvesting step was

repeated if more than 1.5 ml of culture medium was used. 200 µl of PD1 Buffer was added to the tube and the cell pellet was resuspended by pipetting or vortexing. Cultured cells were disrupted by adding 200 µl of PD2 Buffer to each tube and mixing gently by turning the tube upside down for 10 times to avoid shearing of DNA. The tube was placed at room temperature for 2 min until the lysate is homologous. Then 300 µl of PD3 Buffer was added and mixed immediately by inverting the tube gently for 10 times. After centrifugation for 3 min at maximal speed, the supernatant of the cell culture was transferred into a PD column sitting inside a 2 ml collection tube and centrifuged for 30 s. The flow-through was discarded and the PD column was placed back in the same 2 ml collection tube. 400µl of W1 Buffer was added into the PD column and centrifuged for 30 s. After discarding the flow-through and placing the PD column back into the collection tube, 600 µl of Wash Buffer with ethanol added was pipetted into the PD column and centrifuged for 30 s. The PD column was put back in the collection tube and centrifuged for another 3 min to dry the column matrix, after the flow-through is discarded. Subsequently, the PD column was moved to a new Eppendorf tube. DNA elution was then performed by adding 50 µl of sterile water directly into the center of the column matrix and standing for 2 min, before centrifuging for 2 min at maximal speed.

2.10.4 Verification of Constructs by Sequencing

Constructs were sequenced for both strands of the insert region by the dideoxy method (Sanger et al., 1977) using ABI PRISM BigDye^{TM} Cycle Terminator Sequencing Ready Reaction Kit (Applied Biosystems, USA). Sequencing reaction was set by mixing 150-200 ng of double-stranded DNA miniprep as template, 1.6 pmol of sequencing primers, and 4 µl of Terminator Read Reaction Mix, and was then adjusted by deionised water to a final volume of 10 µl. Thermal cycler was programmed to run for 25 cycles of denaturing at 96°C for 10 s, annealing at 50°C for 5 s and extending at 60°C for 4 min. Precipitation of the amplified PCR products were then performed at room temperature for 15 min in 80 µl of 75% isopropanol and followed by centrifugation for 20 min at 14,000 rpm. The cell pellet obtained was washed twice with 500 µl of 75% isopropanol, dried by exposure to air and dissolved in 4 µl of loading buffer which contains 25 mM EDTA (pH 8.0) and formamide. Each sample was denatured by heating for 2 min at 95°C and chilled on ice, before being loaded. ABI PRISM 377 DNA Sequencer (Applied Biosystems, USA) was used for sequencing. Sequences obtained thereby were subsequently blasted within the genome database at the National Centre for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) or the Arabidopsis Information Resource (TAIR, www.arabidopsis.org).

2.11 Transformation of *A. tumefaciens* Competent Cells

Transformation of *Agrobacterium tumefaciens* strain GV3101 with miniprep DNA constructs were performed by electroporation method. The frozen stock of 40 µl of GV3101 competent cells from -80°C freezer were thawed on ice, mixed gently with 2 µl miniprep DNA constructs by pipetting or tapping tube, and placed on ice for another 30 min before loaded into a 1mm prechilled cuvette. Moisture on the outside of the cuvette was wiped off, before the cuvette was placed in an electroporator, Gene Pulser (BioRad, USA), which was set up to use 25 µF, 2.5 kV, 200Ω. Electroporation was performed in pulses with a field strength of 25 kV/cm. The cell suspension was then transferred to an Eppendorf tube with 1 ml of LB medium and cultured for 3-4 hr at 28°C in a shaker (250 rpm). Cultured cells were precipitated by centrifugation at 3000 rpm, and cell pellets were resuspended and plated onto LB agar plates supplemented with 25µg/ml gentamycin, 25µg/ml rifampicin, 10 µg/ml tetracycline, and 50 µg/ml kanamycin. Incubation of the plates continued for 48 hr at 28°C. Individual colonies were selected and resuspended in water. Verification of transformation was performed by colony PCR using a gene-specific primer together with a vector-specific primer. Confirmed colonies were selected to be used for further plant transformation.

2.12 Plant Transformation

Transformation of *Arabidopsis* plants with constructs carrying cloned DNA inserts was achieved by *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). *Agrobacterium* strain GV3101 transformed by desired constructs were cultured at 28°C, until the OD_{600} value reached 0.8, in LB medium supplemented with 25µg/ml gentamycin, 25µg/ml rifampicin, 10 µg/ml tetracycline, and 50 µg/ml kanamycin. Cultured cells were then precipitated by centrifugation for 10 min at 4000 rpm and resuspended completely in a solution containing 5% sucrose and 0.015% surfactant Silwet L-77. Inoculation of plants was achieved by dipping and submerging immature floral buds of different stages on the inflorescences into *Agrobacterium* cell suspension for 1 min. The dipping was repeated once more to improve transformation efficiency. Improved yield of transformants was also achieved by subsequent covering of the inoculated plants to maintain humidity and keeping them in dark for 1 day. The dipped plants were then returned to their normal growth conditions and their seeds were later collected as T1 generation, which were grown and screened by 3% BASTA after the emergence of the first rosette leaf.

Chapter 3 Results

3.1 Introduction

In order to identify genes that are regulated by *SVP* in control of flowering time during the floral transition in *Arabidopsis*, we performed a microarray analysis of the loss-of-function *svp-41* mutants at day 9 after germination (unpublished results). Genes that showed dramatic changes in expression in *svp-41* mutants, when compared with wild-type plants at the same age, were selected for further study. Analysis of expression levels by either real-time quantitative PCR or semi-quantitative RT-PCR was performed for these candidate genes as verification of the microarray results. For each of these potential target genes whose expression change in *svp-41* has been confirmed, available insertional mutants were purchased, and artificial miRNA transgenic lines or overexpression lines were also constructed. Phenotypes of flowering time were observed for each line by counting the number of rosette leaves or total leaves one week after bolting. Target genes whose loss-of-function or overexpression lines showed defects in flowering time as compared with wild-type plants were further analyzed in detail.

3.2 Phenotypic Analysis of *svp-41* mutants

It has been reported that the loss-of-function *svp-41* allele of *SVP* results in early flowering phenotype (Hartmann et al., 2000). Plants carrying homozygous *svp-41* alleles also have smaller leaves and other aerial parts, due to the shorter duration of vegetative growth, as compared with wild-type plants. Scanning electron microscopy (SEM) revealed that the size of cells on the adaxial surface of rosette leaves of 8-day-old *svp-41* mutants was comparable to that of wild-type plants (Fig 3). This indicates that cell number, but not cell size, may contribute to the smaller size of rosette leaves in *svp-41* mutants, as compared with wild-type plants.

3.3 Expression Analysis of *svp-41* mutants

By comparing the expression levels of genes in *svp-41* mutants with wild-type plants based on data from microarray analysis (unpublished data), a list of more than 20 genes were initially selected to be checked for genuine change of expression in *svp-41* mutants (Table 1). In order to verify the expression levels of these genes were indeed up- or down-regulated in *svp-41* mutants, seedlings of *svp-41* and wild-type plants from 3 days to 11 days after germination were collected and analyzed using either real-time or semi-quantitative RT-PCR.

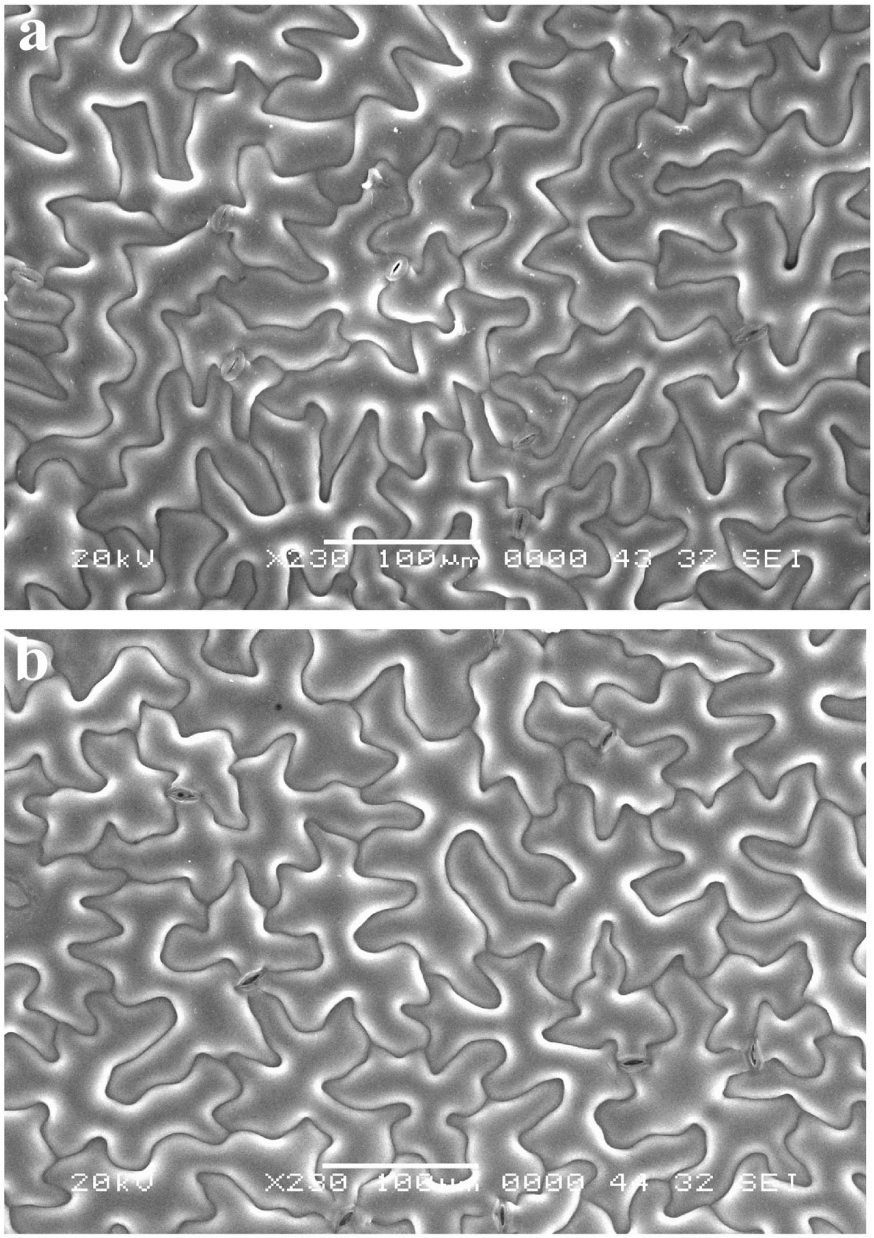

Figure 3. Scanning electron microscopy analysis of adaxial rosette leaves in *svp-41* and wild-type plants. Rosette leaves from 8-day-old *svp-41* (**a**) and wild-type (**b**) plants were collected and frozen with liquid nitrogen, before mounting for SEM observation.

Table 1. List of primer pairs used for real-time PCR analysis

Gene	Sequence
AT1G49620 *KRP7*	5 ’ – TCAGATCTGGAGGCTCATGAAA – 3 ’
	5 ’ – CGCTTGATGAAATTCCCTGTTT – 3 ’
AT1G65620 *AS2*	5 ’ – CCTCTGAGCAACAGAAGCCATTA – 3 ’
	5 ’ – AGTTTGTTGAAGAAGATGCCATTTTA – 3 ’
AT1G70510 *KNAT2*	5 ’ – CAGATGATGGTGCGGTTTCA – 3 ’
	5 ’ – CCGCTGCTATGTCATCATCTTC – 3 ’
AT1G74330	5 ’ – GGGAATGGGAGACGTGGTATAG – 3 ’
	5 ’ – TTTGGTGCCGCACATCTG – 3 ’
AT1G75640	5 ’ – CCGTAACCAAGCTGAGGCTTT – 3 ’
	5 ’ – CAGGTGGTCCGCAGTAATAACC – 3 ’
AT1G77300 *EFS*	5 ’ – GCTGGCGATTTGTGTTCAAA – 3 ’
	5 ’ – CAAGCACCTCTCCAACATATTCAA – 3 ’
AT2G18040 *AtPIN1*	5 ’ – GAAGAGCAAGATGGCGTCGAGAGAC – 3 ’
	5 ’ – CTGCTCAACGGCGGCTTCTCTAG – 3 ’
AT2G26560 *PLP2*	5 ’ – CATCTCTCTGCCGTTTTTCGA – 3 ’
	5 ’ – GCAGCATCCCCAGTTAATGTG – 3 ’
AT3G02820	5 ’ – AGCTAACTCCTGAATTGTTACTCTCTGA – 3 ’
	5 ’ – CTCACTGTACAACCGTATCAAATTCC – 3 ’
AT3G09160	5 ’ – CGGTTTGTTGCTTACCATGGA – 3 ’

	5 ’ – ACAAATGATGCGGCCTTTCT – 3 ’
AT3G13590	5 ’ – TGCAAGCAATGCGACTTTG – 3 ’
	5 ’ – TGTATGGTAAGCGGATGAATATGTATC – 3 ’
AT4G03270 *CYCD6;1*	5 ’ – GCCATTTCTTCAATCACTCAGTATTC – 3 ’
	5 ’ – TGCGGCATATCTTCGCTAGAC – 3 ’
AT4G37630 *CYCD5;1*	5 ’ – CGAAACCCTACCCTCTAAAACGA – 3 ’
	5 ’ – ACCCAAATCTTGTTCTTGTAGTGAGA – 3 ’
AT5G02470 *DPa*	5 ’ – GCAGAAGGATTTACCTTACCATTCA – 3 ’
	5 ’ – AAGTCGAGGTGTACAAGTTGCATATC – 3 ’
AT5G08600	5 ’ – GAGGAAGTAAATCCGAATGACGAA – 3 ’
	5 ’ – CTCTGACCTCTATTGGTTTTACTTACCA – 3 ’
AT5G17490 *RGL3*	5 ’ – TCAGTGGCGAAAACGTATGG – 3 ’
	5 ’ – CGCCAGATAACGCCAATAGC – 3 ’
AT5G55690 *AGL47*	5 ’ – AAGCAGCTGCACCAAGACATAC – 3 ’
	5 ’ – CGTCGGTTTCTCCACCTTAGTG – 3 ’
AT5G58340	5 ’ – GATGTCACTCCTCCGCCTACTC – 3 ’
	5 ’ – CTGCTAGACCATGTTTCCTGACTATC – 3 ’
AT5G66250	5 ’ – ACCGTTACAGGCGTCATGGA – 3 ’
	5 ’ – TTGAATTTCCTCTTGGCTTAGCA – 3 ’
TUB2	5’ – ATCCGTGAAGAGTACCCAGAT – 3’
	5’ – AAGAACCATGCACTCATCAGC – 3’

Genes that were confirmed with significant and consistent changes of mRNA levels in *svp-41* mutants as compared with wild-type plants were chosen to be further studied (Fig. 4). Among these genes, expression levels of AT2G18040 (*AtPIN1*) (Fig. 4a), AT5G66250 (Fig. 4b), and AT3G09160 (Fig. 4c) were found to be significantly lower in *svp-41* mutants than those in wild-type plants continually from 3 days to 9 or even 11 days after germination. Significant reduction in mRNA levels were also observed for AT2G26560 (*PLP2*) at day 3 (Fig. 4d), AT5G02470 (*DPa*) and AT1G49620 (*KRP7*) at day 5 after germination (Fig. 4e, 4f) in *svp-41* mutants as compared with wild-type plants.

3.4 Phenotypic Analysis of Mutants and Transgenic Lines

Loss-of-function lines of verified candidate genes were either purchased as insertional lines or constructed using artificial miRNAs or antisense RNAi.

Overexpression lines for these genes were also constructed by driving each of them with CaMV 35S promoter. Phenotypic analysis of flowering time was conducted by counting the number of rosette leaves or total leaves that include both rosette and cauline leaves, about 1 week after bolting. Among the genes selected for testing, none of the loss-of-function or overexpression lines showed flowering time phenotype (data not shown), except for one gene,

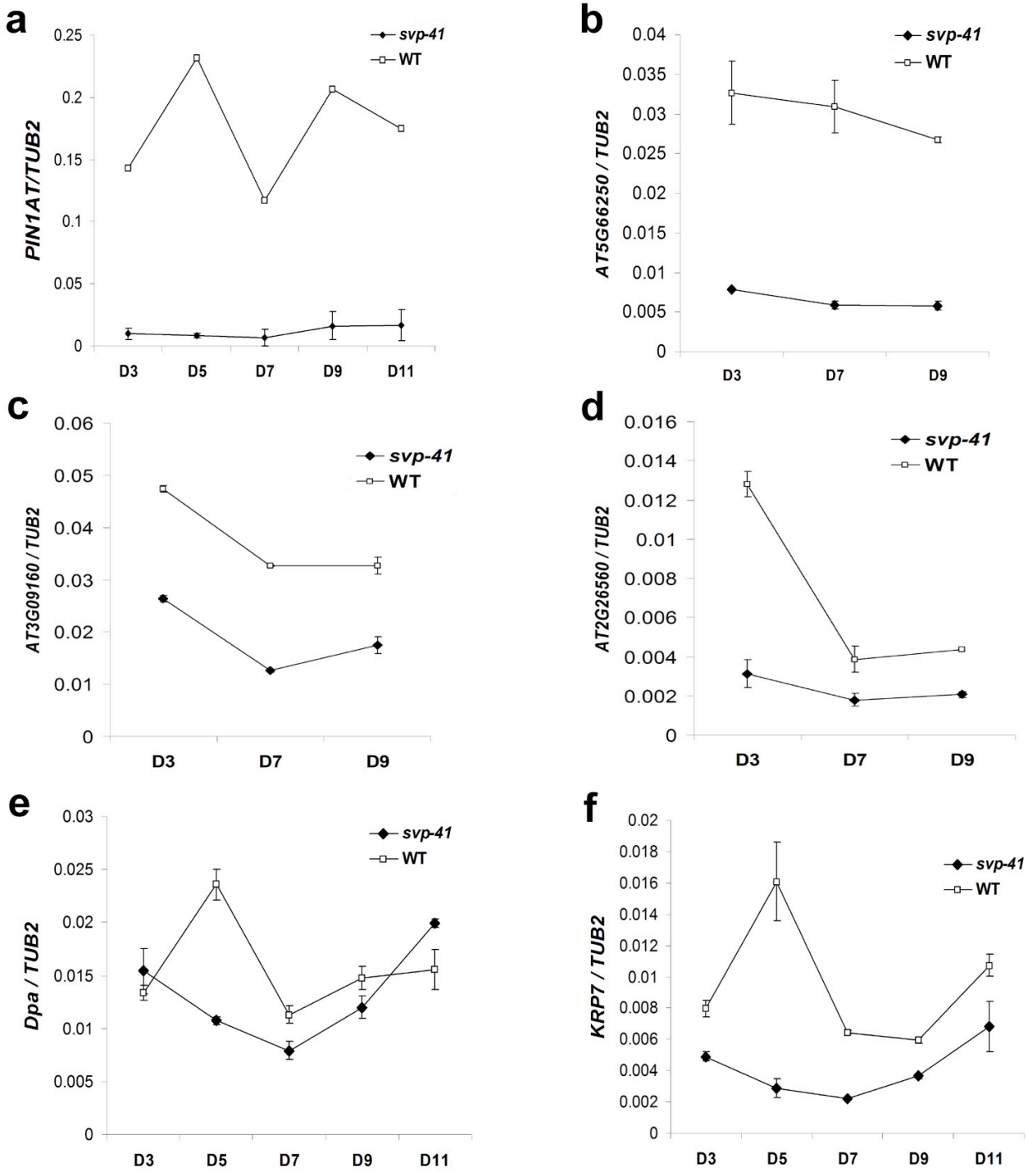

Figure 4. Comparison of gene expression in *svp-41* and wild-type plants. **a.** AT2G18040 (*AtPIN1*), **b.** AT5G66250, **c.** AT3G09160, **d.** AT2G26560 (*PLP2*), **e.** AT5G02470 (*Dpa*), **f.** AT1G49620 (*KRP7*).

*AT2G18804*0, which encodes a parvulin peptidyl-prolyl *cis*/*trans* isomerase, named as *AtPIN1* (Landrieu et al., 2000). Its human ortholog PIN1, specifically recognizes phosphorylated Thr or Ser residue prior to a proline (pSer/Thr-Pro) and catalyzes the *cis*/*trans* conformational changes of the peptide bond between pSer/Thr and Pro (Lu and Zhou, 2007; Yao et al., 2001). Expression of *AtPIN1* is dramatically and consistently reduced in *svp-41* mutants as compared with wild-type plants from 3 days to 11 days after germination (Fig. 4a).

3.5 Phenotypes of *AtPIN1* knockdown and overexpression lines

Transgenic lines in which *AtPIN1* expression was suppressed by antisense RNA flowered later than wild type under both long days and short days (Fig. 5c, d; Fig. 6a, b), while overexpression of *AtPIN1* with the *AtPIN1* cDNA under the control of CaMV promoter resulted in early flowering phenotype under both long days and short days (Fig. 5a, b; Fig 6a, b). *AtPIN1* mRNA levels in both knockdown and overexpression lines were verified using semi-quantitative RT-PCR or northern blot (Fig. 6c, d) (Table 2). These observations show that *AtPIN1* promotes flowering in *Arabidopsis*.

Besides the delayed-flowering phenotype observed for *AtPIN1* antisense lines,

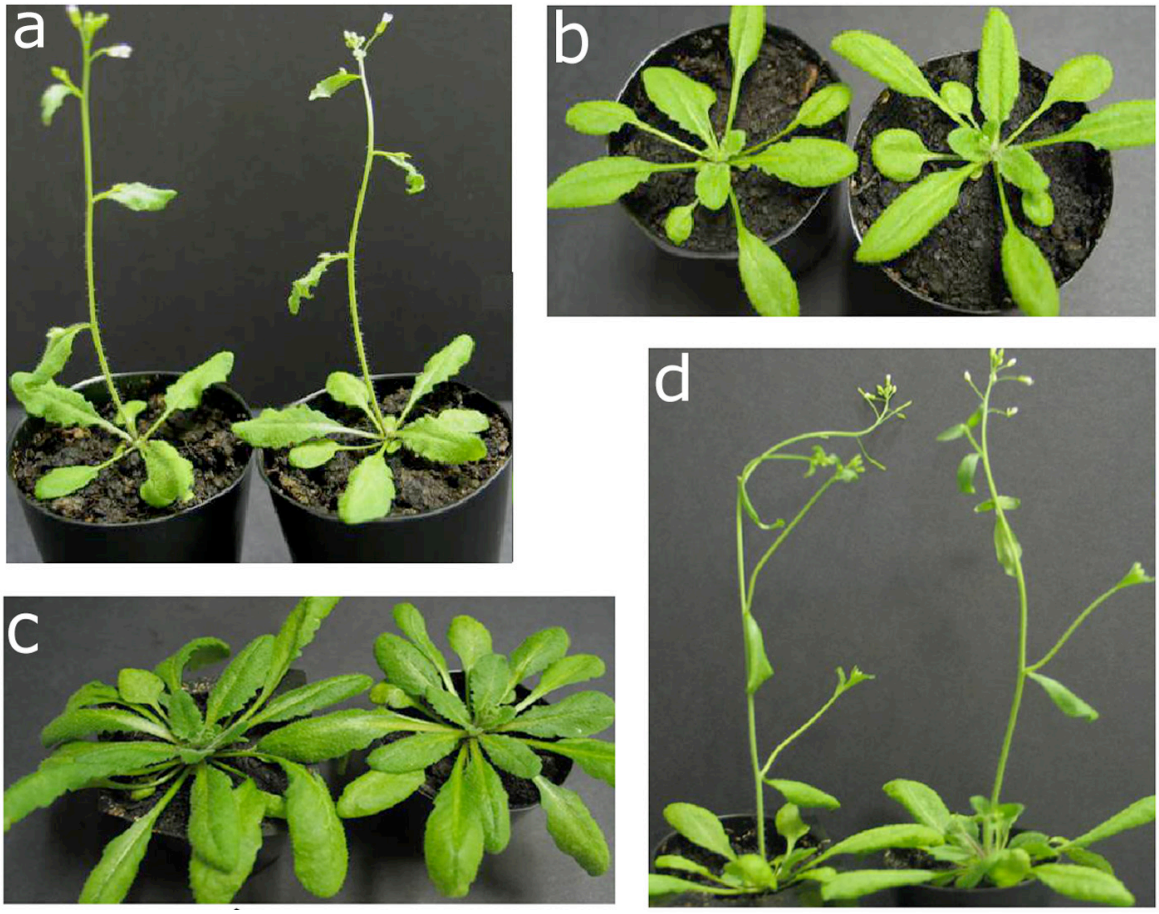

Figure 5. Phenotypes of *AtPIN1* antisense and *35S:AtPIN1* plants. a, b, *35S:AtPIN1* plants plants (**a**) flower earlier than wild-type plants (**b**) at 28 days after germination under long days. **c, d,** *AtPIN1* knockdown plants with RNAi (**c**) show delayed flowering phenotype compared to wild-type plants (**d**) at 35 days after germination under long days.

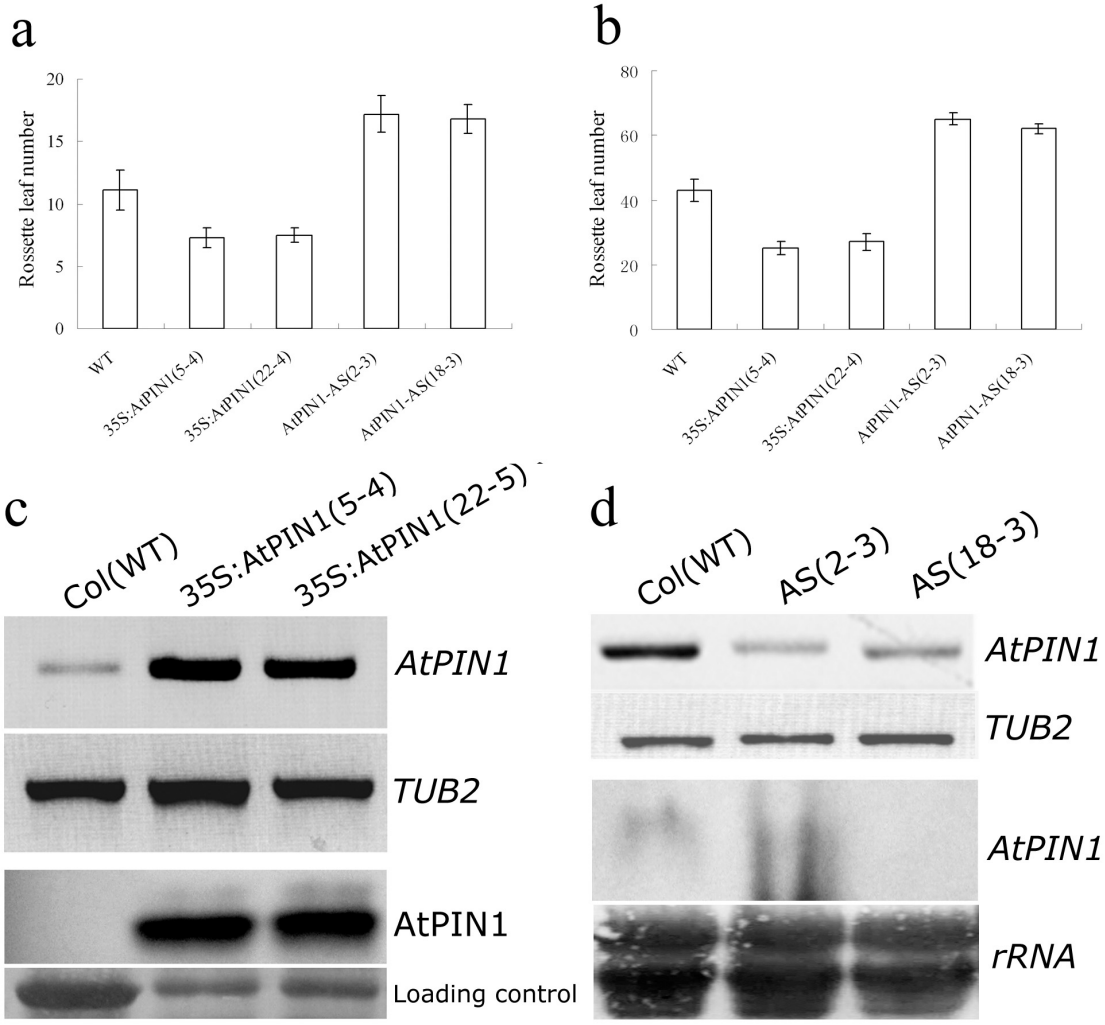

Figure 6. Flowering time of *AtPIN1* transgenic lines and expression of *AtPIN1* in these lines. a, b, Flowering time of *AtPIN1* under long days (**a**) and short days (**b**), measured by the number of rosette leaves on the main shoot. **c, d,** *AtPIN1* expression in its transgenic lines was analyzed, using semi-quantitative RT-PCR together with either Western blot analysis (**c**) or Northern blot analysis (**d**). *TUB2* was used as an endogenous control in RT-PCR, while rRNA levels provide a measure of total RNA loaded in each lane.

Table 2. List of primers used for *AtPIN1*

Primer	Sequence
AtPIN1-35S	5’ – CCGCTCGAGATGGCGTCGAGAGAC – 3’
	5’ – CGCTCTAGACGAAGCAGGCACCTT – 3’
AtPIN1-antisense	5’ – CGCTCTAGAATGGCGTCGAGAGAC – 3’
	5’ – CGCTCGAGAGCAGAGCTACAGTCA – 3’
AtPIN1-amiRNA	5’–GATAAGATGCCAGAATGTTGCACTCTCTCTTTTGTATTCC–3’
	5’–GAGTGCAACATTCTGGCATCTTATCAAAGAGAATCAATGA–3’
	5’–GAGTACAACATTCTGCCATCTTTTCACAGGTCGTGATATG–3’
	5’–GAAAAGATGGCAGAATGTTGTACTCTACATATATATTCCT–3’
AtPIN1 quantitative real-time PCR	5’ – GAAGAGCAAGATGGCGTCGAGAGAC – 3’
	5’ – CTGCTCAACGGCGGCTTCTCTAG – 3’
AtPIN1 semi-quantitative RT-PCR	5’ – TAAGGCATCGCACATTTTGA – 3’
	5’ – TGGACTCCACTGTCTGTATCG – 3’

a defect in fertility was also observed in a *AtPIN1* knockdown line using WMD-designed artificial microRNA (Schwab et al., 2006). In the *AtPIN1* amiRNA lines, we observed defects of stamen growth that resulted in failure of pollinating the carpels (Fig 7).

3.6 Genetic Cross Analysis of *AtPIN1*

In order to test whether the promotive function of *AtPIN1* in flowering is mediated by the floral pathway integrators, we performed genetic cross using *AtPIN1* transgenic lines (Fig. 8). While down-regulation of *AtPIN1* had no effect on the early flowering phenotype of *35S:CO* or *35S:FT*, it delayed the early flowering of *35S:SOC1* and *35S:AGL24* (Fig. 8b). This suggests that the function of *SOC1* and *AGL24* in promoting flowering is partially dependent on AtPIN1 activity. *35S:AtPIN1* was able to promote flowering in *co-1* mutants, but only slightly affected flowering in *soc1-2* and *agl24-1*, and almost had no effect on *soc1-2 agl24* double mutants, implying that *SOC1* and *AGL24* are the mediators of *AtPIN1* in promoting flowering (Fig. 8a). Analysis of *SOC1* and *AGL24* expression in transgenic plants that either over-express or suppress *AtPIN1* revealed that their mRNA levels were independent of AtPIN1 activity (Fig. 9a). On the other hand, the expression of *AtPIN1* was also not significantly influenced by AGL24 and SOC1 (Fig. 9b). Taken together, these observations imply that *AtPIN1* and the two flowering regulators, *SOC1* and

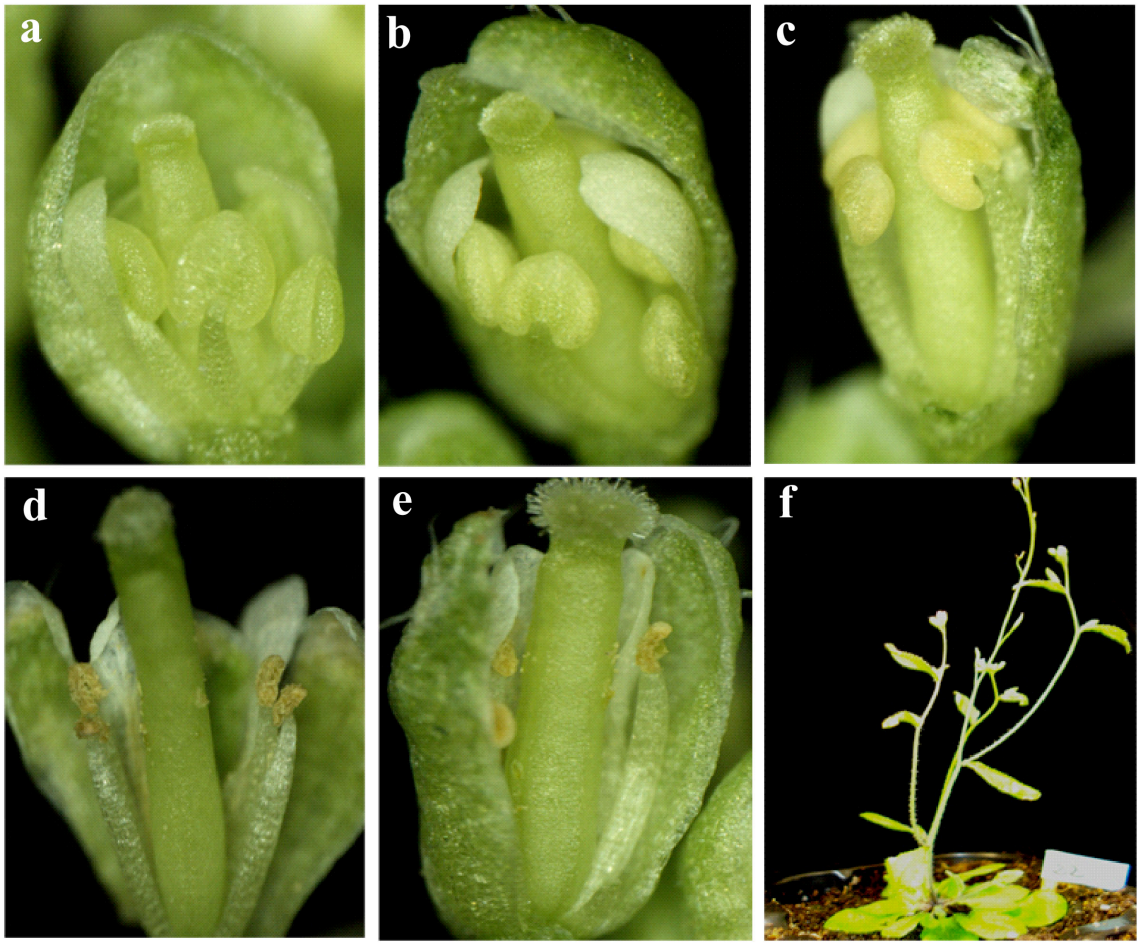

Figure 7. Infertility phenotype of an *AtPIN1* knockdown line using amiRNA. a – e, Defective stamens of three-week-old seedlings whose *AtPIN1* expression was knocked down by amiRNA fail to pollinate carpels. **f,** A three-week-old *AtPIN1* amiRNA line.

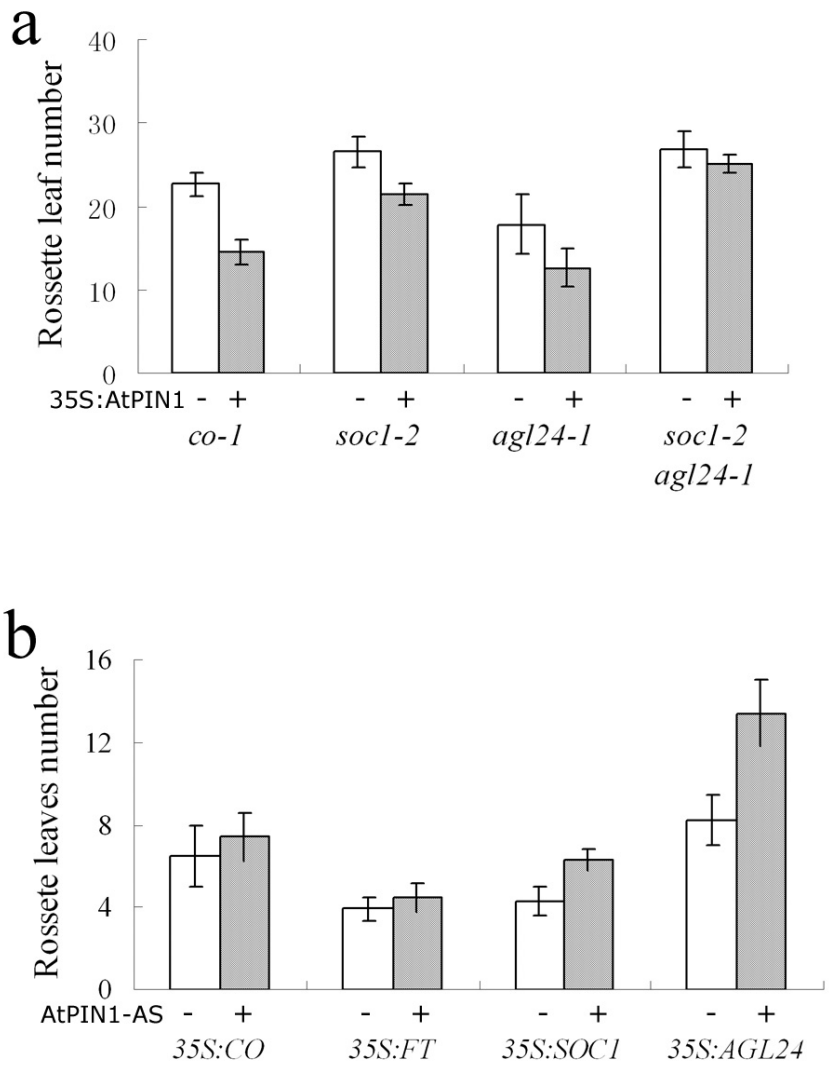

Figure 8. Genetic cross analysis of *AtPIN1* transgenic lines. a, Flowering time of crosses between *35S:PIN1At* and loss-of-function mutants of various flowering promoters under long days. **b,** Flowering time of crosses between *AtPIN1* knockdown lines and overexpression lines of various flowering promoting genes under long days.

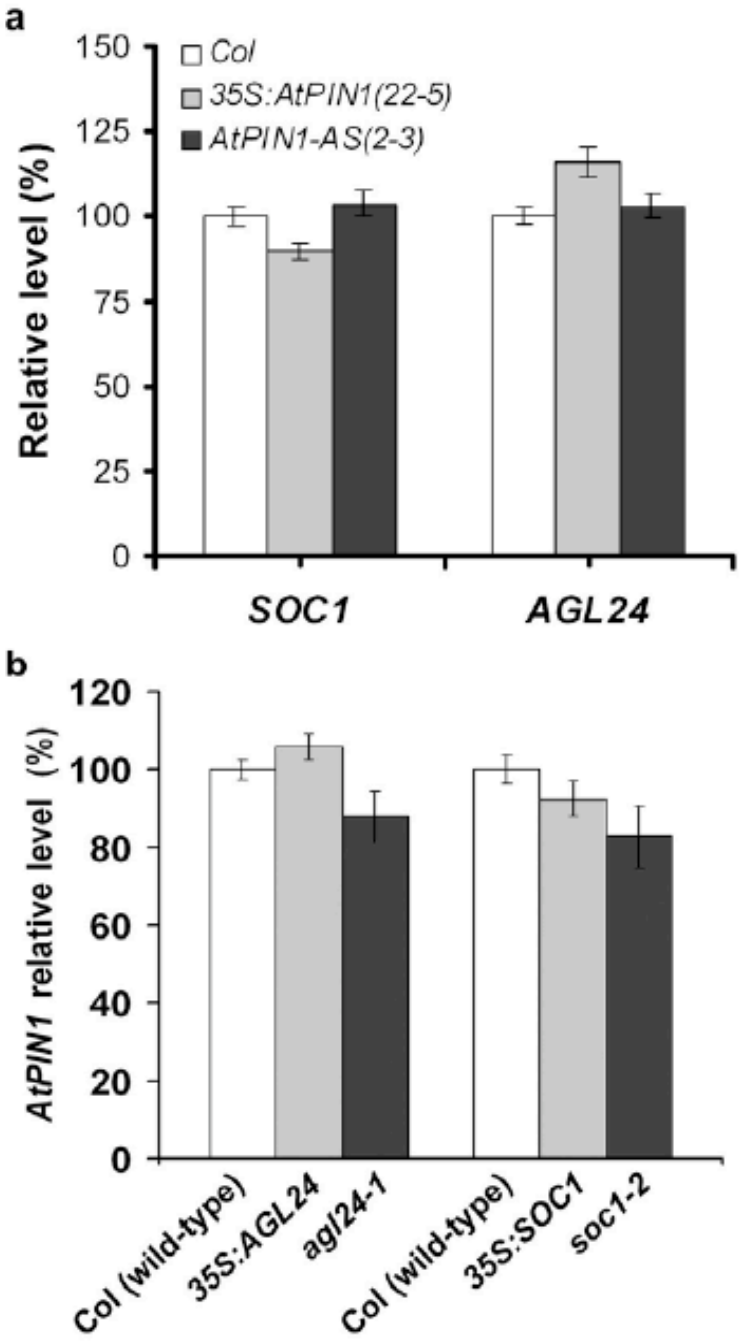

Figure 9. Relationship of *AtPIN1* with *SOC1* and *AGL24*. **a,** Expression of *SOC1* and *AGL24* in 9-day-old *AtPIN1* transgenic lines. **b,** Expression of *AtPIN1* in 9-day-old plants with ectopic or missing activity of *SOC1* and *AGL24*. *TUB2* expression was used for normalization purpose.

AGL24, function together to promote flowering in a mutually dependent manner and this synergistic interaction occurs on a post-transcriptional level.

3.7 ChIP Assays of *AtPIN1* promoters

To examine whether SVP directly regulates *AtPIN1* expression, ChIP assays were performed using a previously reported functional transgenic line *svp-41 SVP:SVP-6HA*, which contains *HA*-tagged *SVP* controlled by its endogenous promoter (Li et al., 2008). We scanned the *AtPIN1* genomic region for CArG box motifs and designed five primer pairs near the identified motifs to measure potential enrichment of DNA fragments (Fig. 10a; Table 3). Eight-day-old seedlings of *svp-41 SVP:SVP-6HA* and wild type were harvested to be immunoprecipitated with *HA* antibody, and further analyzed with real-time PCR. Relative enrichment fold was calculated by first normalizing all values against a genomic fragment of *ACTIN*, and then comparing the value of transgenic plants to that of the wild-type plants. Fragment 4, which flanks a CArG box motif in the second exon near the C-terminus on the *AtPIN1* genomic region, was slightly enriched as compared with fragment 3, which flanks a CArG box motif in the intronic region. However, no significant enrichment was observed when enrichment of fragment 4 was compared to the other fragments, indicating weak or none binding of SVP-6HA protein to fragment 4.

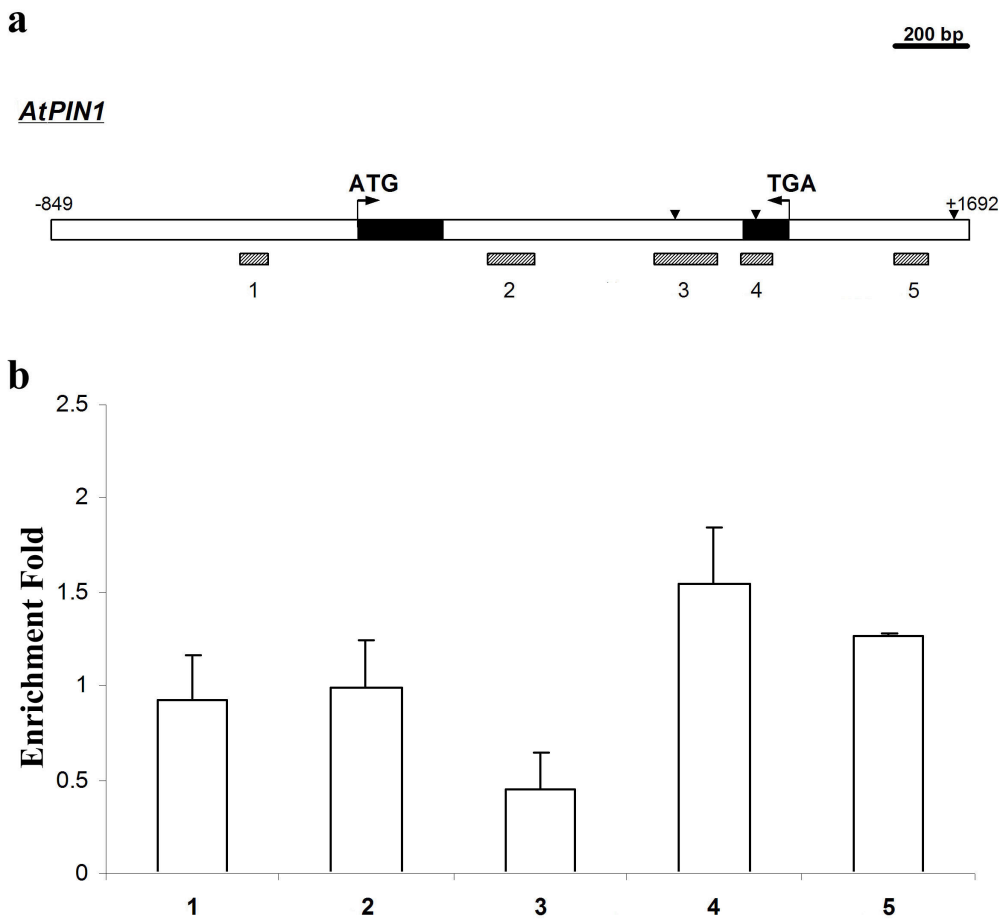

Figure 10. ChIP analysis of *AtPIN1* promoter. a, Schematic diagram of the *AtPIN1* genomic region. Black boxes represent exons, while white boxes represent either introns, or upstream or downstream regions. Arrowheads indicate the sites containing either a single mismatch or a perfect match to the CArG motif sequence. Five DNA fragments flanking these sites were tested for enrichment. **b,** ChIP enrichment test of SVP-6HA binding to *AtPIN1* promoter region. Eight-day-old seedlings of *svp-41 SVP:SVP-6HA* and wild type were harvested for ChIP assays. To calculate the relative enrichment of each fragment, the fragment amounts were first normalized against a genomic fragment of *ACTIN*, and then the value for transgenic plants were normalized against that for wild-type as a negative control.

Table 3. List of primers used for ChIP assays

Primer	Sequence
AtPIN1 ChIP-1	5’ – GATCCGAGGCGGTTTCATC – 3’
	5’ – TTCAAAATGCTCCCGGATCT – 3’
AtPIN1 ChIP-2	5’ – GTTTGTTGAAGGAGTAGAAAGCTTGTT – 3’
	5’ – ACATCGAGGAAGAACTGTGAGATTC – 3’
AtPIN1 ChIP-3	5’ – CTGTTTGCTTAGAGCATAACTATGTTT – 3’
	5’ – GGGAGATGATCTACGAAGATGTTAGTA – 3’
AtPIN1 ChIP-4	5’ – CCTGTTCAGGTTCCTTTGGTAGA – 3’
	5’ – TGTCGCTTATATCTCCAACCTTGA – 3’
AtPIN1 ChIP-5	5’ – CATCTTGTGTGTTGTCATTTTGCT – 3’
	5’ – CGCCTAACAGTAAACGATTTCTCA – 3’

3.8 Flowering Pathway Analysis of *AtPIN1*

In order to define the position of *AtPIN1* within the network of flowering pathways, its expression in wild types and various flowering mutants was examined. An ascending trend of *AtPIN1* expression was observed in seedlings from 9 to 13 days after germination under long days, but not short days, indicating the *AtPIN1* responds to the photoperiod pathway (Fig 11b). *AtPIN1* expression was also found to be affected by vernalization, but was comparable in wild-type plants and *FRI FLC* plants with or without vernalization, suggesting that *AtPIN1* is regulated by the vernalization pathway in a *FLC*-independent manner (Fig. 11b). GA and autonomous pathways seem to have no effect on *AtPIN1* expression (data not shown).

3.9 *AtPIN1* Expression Pattern Analysis

Spatial expression pattern of *AtPIN1* was revealed by *in situ* hybridization method. *AtPIN1* mRNA were found to be localized in the shoot apical meristem and emerging leaves in 9-day-old wild-type (Fig. 12a, b, c) and *svp-41* (Fig. 12d, e, f) plants. The concomitant accumulation of *AtPIN1* mRNA with that of *SOC1* and *AGL24* in the shoot apical meristem during floral transition further supports the hypothesis that they may function together through protein interactions to control flowering time.

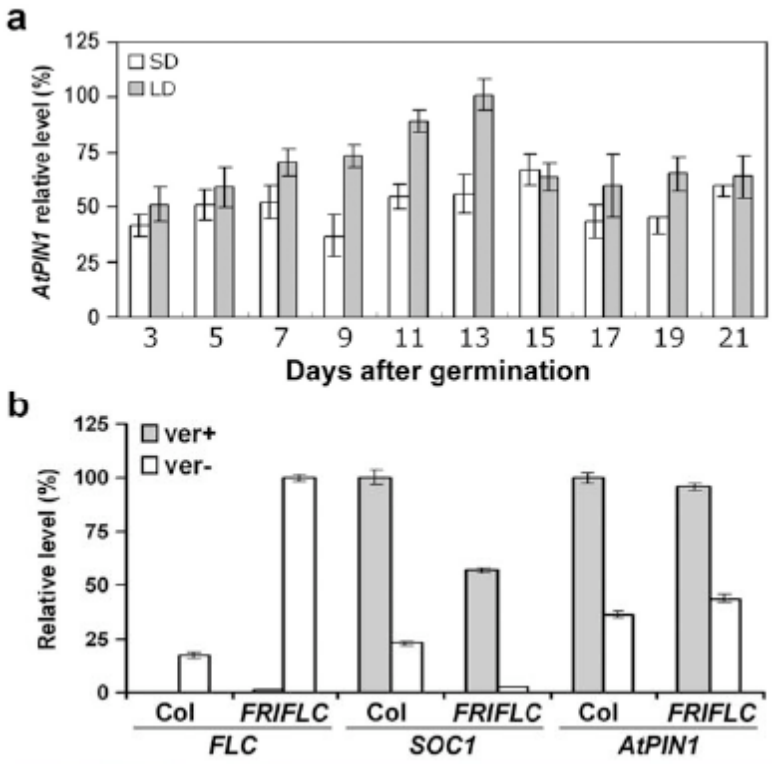

Figure 11. Flowering pathway analysis of *AtPIN1*. **a,** Temporal expression of *AtPIN1* in wild-type plants grown under long days (LD) and short days (SD). **b,** Expression of *AtPIN1* with or without vernalization treatment, which was performed by incubating seeds sown on Murashige and Skoog (MS) agar plates at 4°C under low light conditions for 8 weeks.

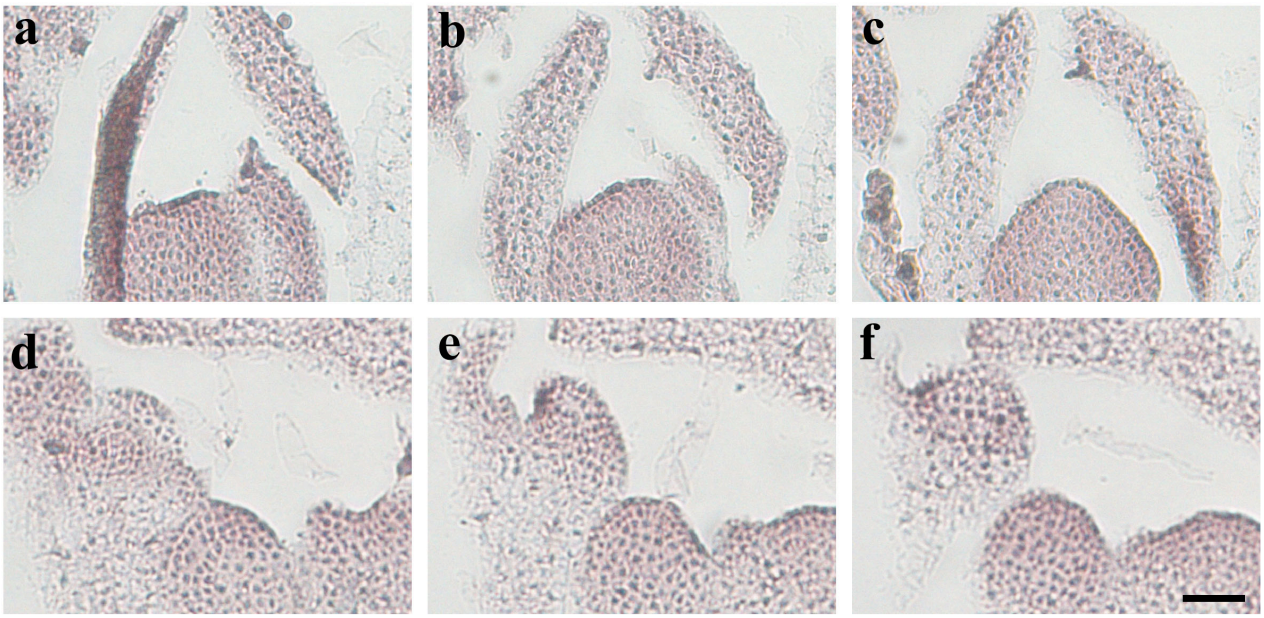

Figure 12. *AtPIN1* expression patterns in wild-type and *svp-41* plants. **a - f,** Localization of *AtPIN1* mRNA by *in situ* hybridization during floral transition. Shoot apices of 9-day-old wild-type (**a** – **c**) and *svp-41* (**d** – **f**) plants were sectioned and hybridized with the *AtPIN1* antisense probe. Scale bar: 25 µm.

Significant reduction of *AtPIN1* expression level was not observed in the shoot apical meristems of *svp-41* mutants, as compared with wild-type plants. This indicates that repression of *AtPIN1* mRNA expression in *svp-41* mutants may occur in tissues other than the shoot apical meristems, such as mature leaves.

3.10 Sequence Alignment of AtPIN1 with Its Homologs

Alignment of amino acid sequence of *Arabidopsis* PIN1 (AtPIN1) and its multiple homologs from *E. coli* (EcPIN1), *Drosophila* (DmPIN1), human (HsPIN1), apple (MdPIN1), and yeast (ScESS1), was performed using ClustalW2 software (Chenna et al., 2003; Larkin et al., 2007). Residues that are shaded in black or gray represent identity or similarity to the column consensus (Fig. 13). Although AtPIN1 lacks the WW domain that is conserved in non-plants eukaryotes, its substrate specificity to recognize phosphorylated Ser/Thr residues preceding proline (pSer/Thr-Pro) is conserved with its homologs (Landrieu et al., 2000).

In mammals, one of the *PIN1* functions is to regulate the transcription of the cell cycle arrest genes, $p21^{Cip1}$ and $p27^{Kip1}$ (Brenkman et al., 2008; Wulf et al., 2002), which are members of the Cip/Kip family. Cip/Kip family is one of the two subfamilies of the cyclin-dependent kinase inhibitors (CKIs), with the other one called INK4 family (Pavletich, 1999). The cyclin-dependent kinases

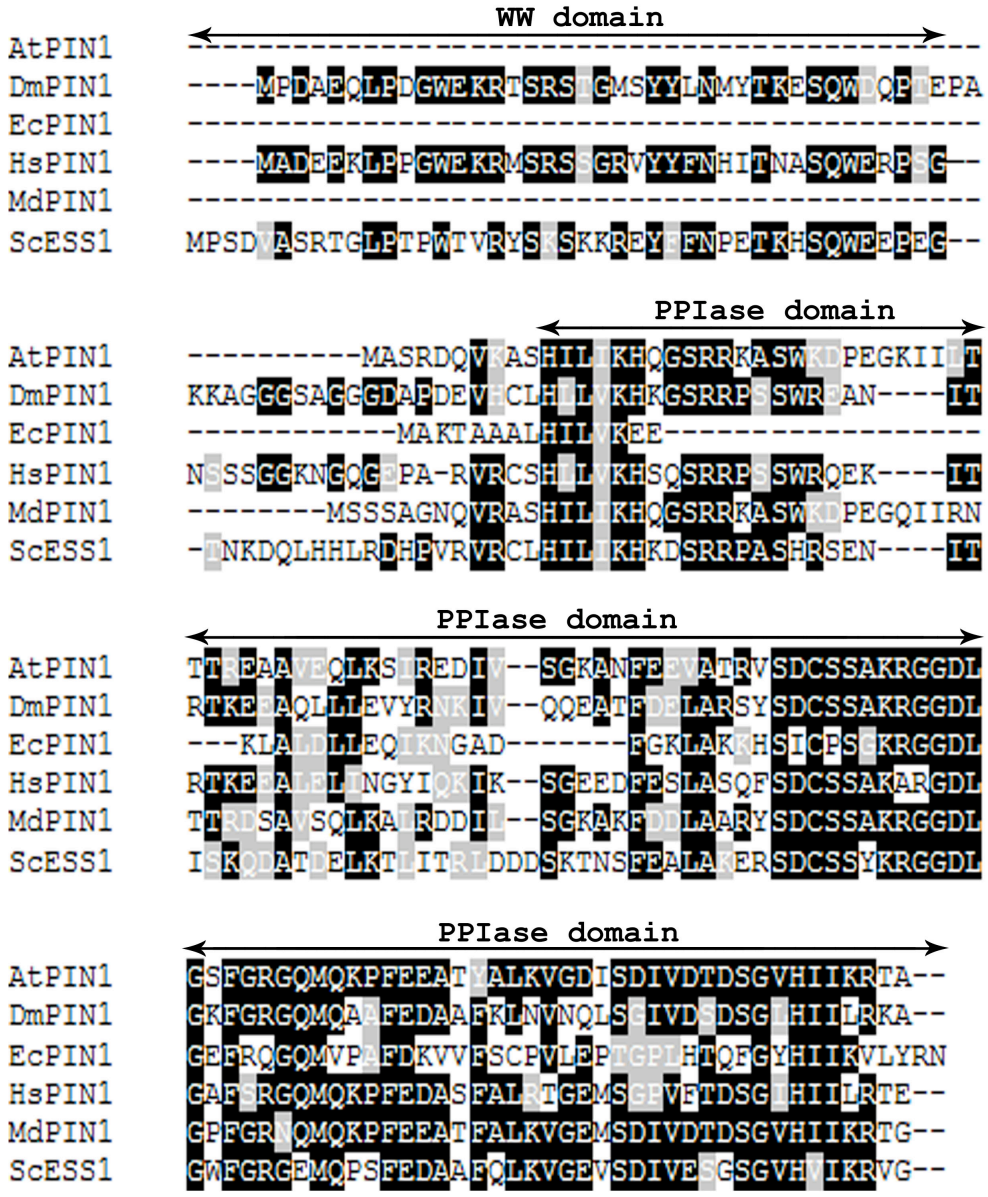

Figure 13. Sequence alignment of AtPIN1 and its homologs. Amino acid sequence of *Arabidopsis* PIN1 (AtPIN1) was aligned with sequences of its multiple homologs from *E. coli* (EcPIN1), *Drosophila* (DmPIN1), human (HsPIN1), apple (MdPIN1), and yeast (ScESS1). Residues that are shaded in black or gray represent identity or similarity to the column consensus.

(CDK) are activated by binding to a regulatory cyclin subunit, and together controls the cell cycle (Pines, 1999). The Cip/Kip family is proposed to control the G1/S and G2/M transitions by forming inhibitory complexes with different cyclin/CDK complexes (Nakayama and Nakayama, 1998).

In *Arabidopsis*, seven genes that show sequence similarity to $p27^{Kip1}$ have been identified and termed Kip-related proteins 1 – 7 (KRP1 – KRP7) (De Veylder et al., 2001; Lui et al., 2000; Wang et al., 1997; Zhou et al., 2002). In an attempt to examine whether a similar regulatory relationship between *AtPIN1* and *KRPs* is conserved in *Arabidopsis*, we performed molecular characterization of *KRP1* and *KRP2*.

3.11 Expression Analysis of *KRP1* and *KRP2*

Examination of *KRP1* and *KRP2* expression levels in various flowering mutants showed that expression of both *KRP1* and *KRP2* was slightly up-regulated in *five* mutants under Ler background (Fig. 14a), and in *agl24*, *ft*, and *FRI FLC* mutants under Col background (Fig. 14b). Notably, *KRP1* expression was also increased in *fca* (Ler) mutants, and slightly in *ft* (Ler), *agl24* (Ler), *co* (Col) and *gi* (Col) (Fig. 14a, b), while *KRP2* expression was slightly increased in *ft* (Col) mutants (Fig. 14b).

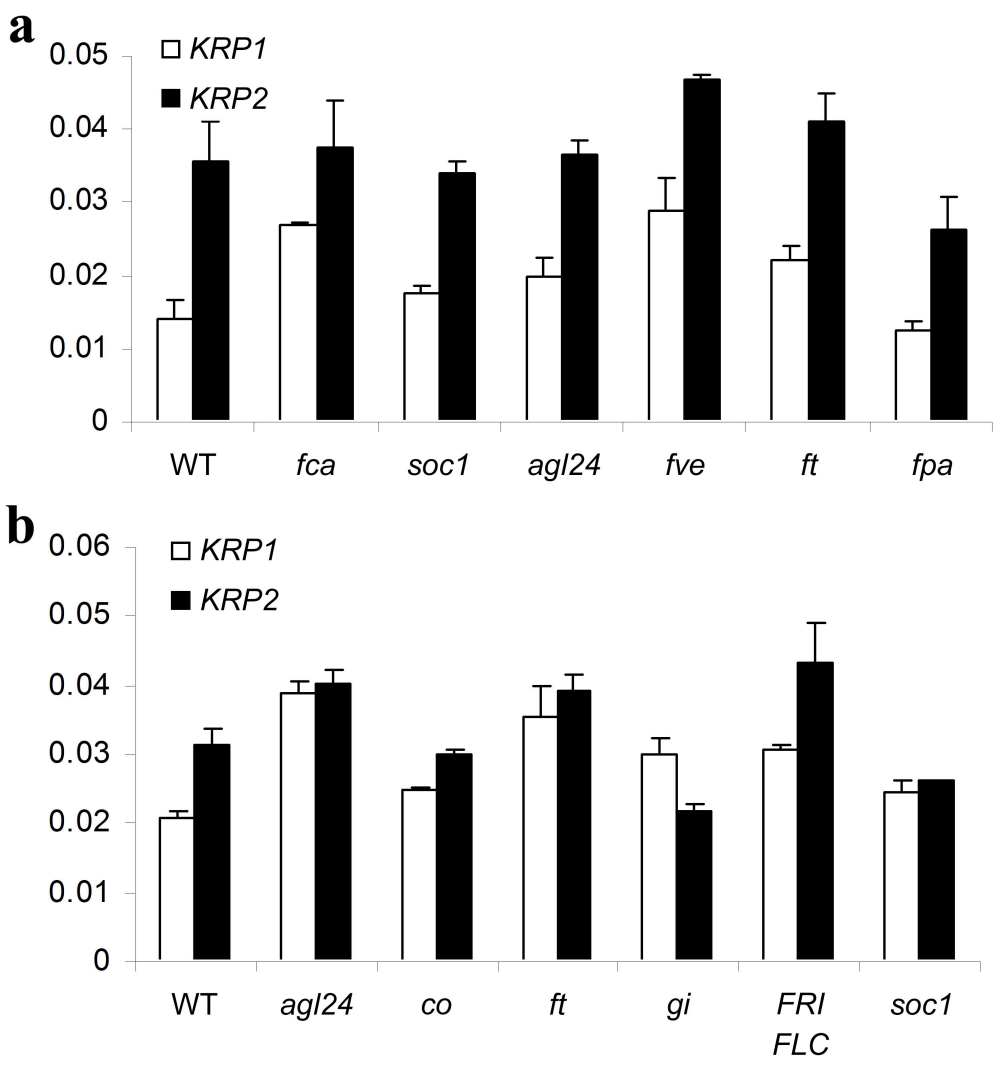

Figure 14. Analysis of *KRP1* and *KRP2* expression in various flowering mutants. **a, b,** Expression analysis of *KRP1* and *KRP2* using quantitative real-time PCR. Total mRNA was extracted from 13-day-old mutants under L*er* background (**a**) and Col background (**b**). All expression values were normalized according to *TUB2* mRNA level, which was used as an endogenous control.

Temporal expression of *KRP1* and *KRP2* was also examined in both Ler and Col wild-type plants, under long days and short days, respectively (Fig. 15). The results showed that *KRP2* expression increased from day 3 to day 7, and decreased afterwards under both long days and short days (Fig. 15a), while *KRP1* expression remained almost constant under both conditions (Fig. 15b).

Comparison of GA-treated wild-type Col plants with mock-treated plant grown under short days revealed that *KRP1* expression was consistently and significantly repressed by GA application during the first four weeks (Fig. 16a), while *KRP2* expression was only slightly decreased in the second week (Fig. 16b). Analysis of vernalized wild-type and *FRI FLC* plants showed that both *KRP1* and *KRP2* were upregulated in response to vernalization treatment (Fig. 17).

Taken together, these expression results indicate that expression of *KRP1* and *KRP2* may be modulated by the autonomous and vernalization pathway, while *KRP1* may also be regulated by the GA pathway.

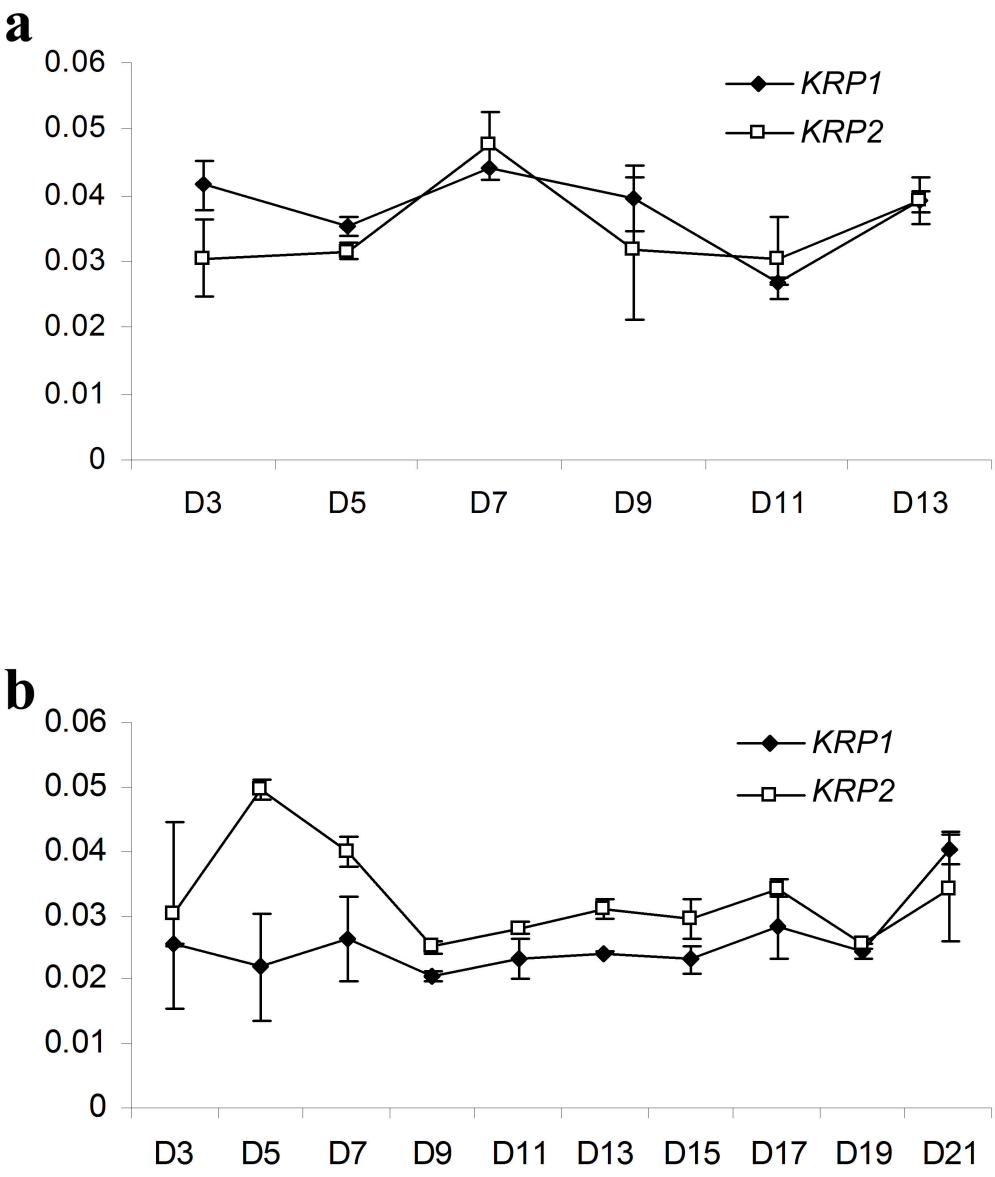

Figure 15. Analysis of *KRP1* and *KRP2* expression in long days and short days. a, Wild-type plants with L*er* background from 3-day-old to 13-day-old plants under long days were collected and analyzed. **b,** Col wild-type plants from 3-day-old to 21-day-old under short days were used for analysis of *KRP1* and *KRP2* expression. All expression values were normalized according to the mRNA level of an endogenous control, *TUB2*.

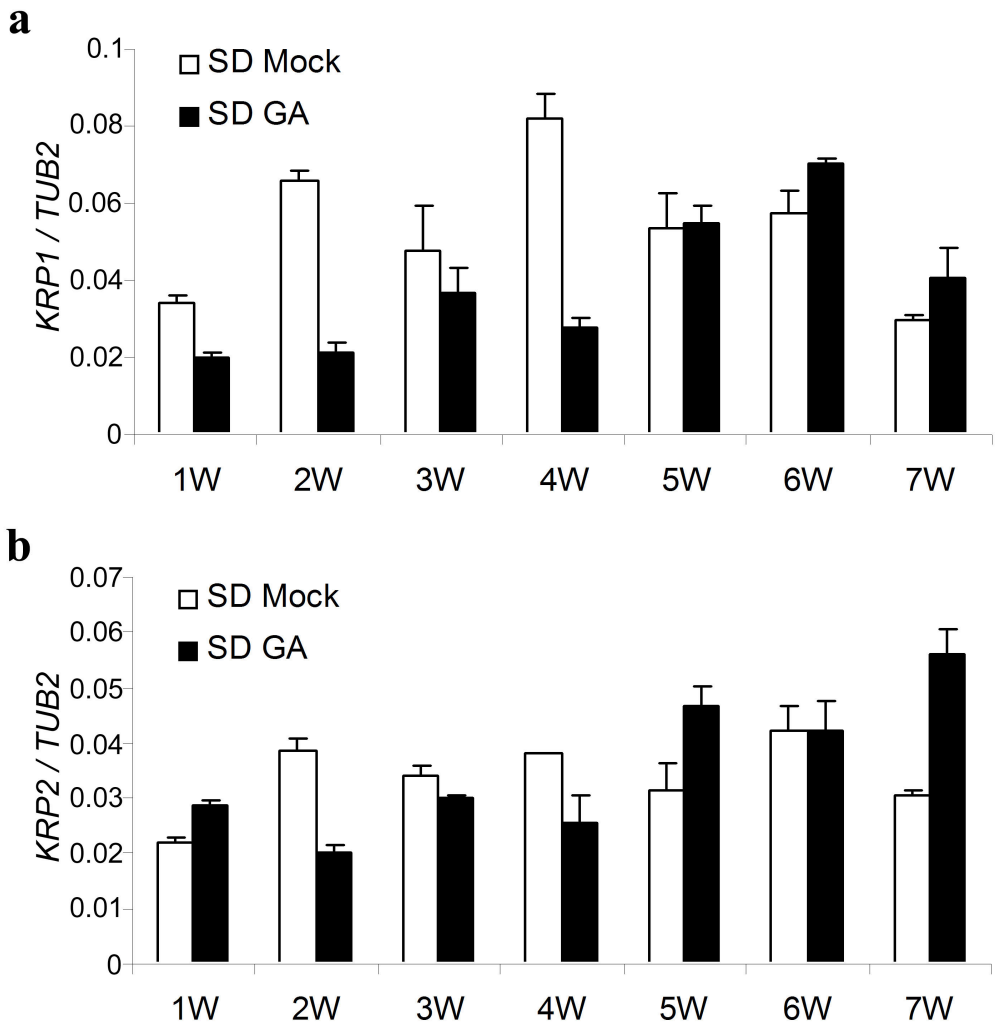

Figure 16. Analysis of *KRP1* and *KRP2* expression under GA treatment. **a, b,** Exogenous GA was sprayed at a concentration of 100 μM weekly onto wild-type plants grown under SDs. Seedlings from week 1 to week 7 were harvested for analysis of *KRP1* (**a**) and *KRP2* (**b**) expression.

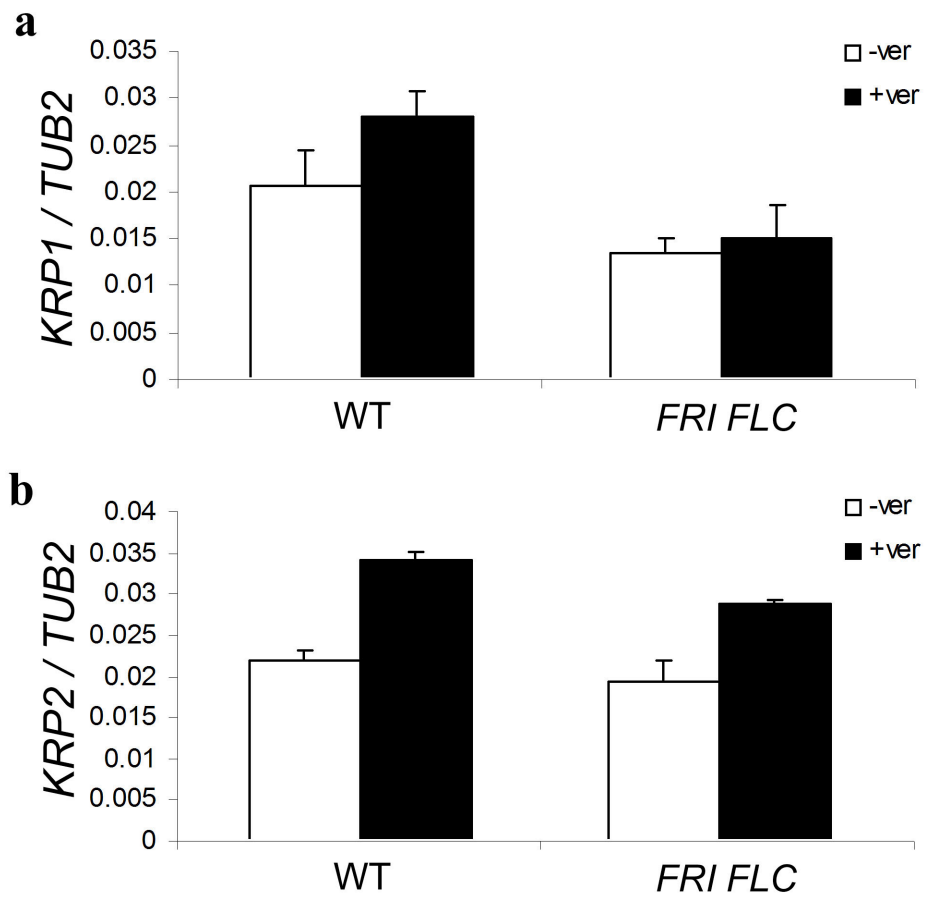

Figure 17. Analysis of *KRP1* and *KRP2* expression under vernalization treatment. **a, b,** Vernalization treatment was applied by incubating seeds sown on Murashige and Skoog (MS) agar plates at 4°C under low light conditions for 8 weeks. The seeds were then grown under normal conditions and harvested at 9-day-old for analysis of *KRP1* (**a**) and *KRP2* (**b**) expression.

Chapter 4 Discussion

Multiple flowering pathways cooperate with each other to regulate the floral transition in plants in response to diverse endogenous and environmental signals. A number of flowering time regulators have been identified to be involved in this dynamic process, among which FLC has been proposed to be a central repressor of flowering promoting pathways by antagonizing the activation of the floral pathway integrators (Boss et al., 2004; Reeves and Coupland, 2001). Recent findings suggest that SVP is another central repressor that mainly responds to internal flowering signals and interacts with FLC in a mutually dependent manner (Li et al., 2008). SVP and FLC function together in response to converged flowering signals from autonomous, GA, and vernalization pathways to regulate the expression of floral pathway integrators, *SOC1* and *FT* (Li et al., 2008). The observation that the introduction of loss-of-function *svp-41* allele into *soc1 ft* double mutant was able to rescue the late flowering phenotype of the double mutant (Li et al., 2008), suggests that there are more target genes besides *SOC1* and *FT* that are transcriptionally regulated by *SVP* in control of flowering time. These genes are yet to be identified. In this study, we searched for *SVP* target genes based on our knowledge of genes that are up- or down-regulated in *svp-41* mutants as compared with wild-type plants, obtained from a previously performed

microarray analysis. We have found that the *Arabidopsis* prolyl isomerase gene, *AtPIN1*, is transcriptionally regulated by SVP, and promotes flowering in a manner that depends on the activity of SOC1 and AGL24 at a post-transcriptional level. Its spatial and temporal expression domains coincide with that of *SOC1* and *AGL24*, indicating that AtPIN1 could directly interact with phosphorylated SOC1 and AGL24.

AtPIN1 was identified as the first PIN1-type parvulin from *Arabidopsis* that catalyzed *cis*/*trans* conformation conversion of peptidyl prolyl in a phosphorylation-dependent manner (Landrieu et al., 2000). It is well conserved in many other organisms (Fig. 13). Its orthologs, such as Protein Interacting with NIMA-1 (PIN1) from human (Lu et al., 1996), and Essential (ESS1)/Processing/Termination Factor 1 (PTF1) from *Saccharomyces cerevisiae* (Hanes et al., 1989; Hani et al., 1995), have been proposed as novel molecular timers that regulate the amplitude and duration of a given cellular response or process(Lu and Zhou, 2007). These enzymes, as well as AtPIN1, recognize only phosphorylated Ser/Thr residues preceding proline (pSer/Thr-Pro) that normally takes one of two distinct conformations: *cis* and *trans* (Hani et al., 1999; Yaffe et al., 1997). By interacting with phosphorylated substrates as described, PIN1 homologs are able to catalyze their conformational changes and thereby regulate their biological function (Liou et al., 2003).

There is one pThr-Pro at the C-terminus of AGL24, and two pSer-Pro located near each end of SOC1 (Fig. 18). All of these are all potential binding sites for AtPIN1 protein. Therefore, it is likely that the role of AtPIN1 in promoting flowering is mediated by its ability to catalyze the conformational change of the pSer/Thr-Pro motifs within SOC1 and AGL24, therefore leading to accumulation of specific conformational forms of SOC1 and AGL24 that may function as a switch and promote the floral transition from vegetative growth to reproductive growth.

The Ser/Thr-Pro motif can also be found in many other flowering time regulators, as well as flower development regulators, such as AG, AP1, AP3, CAL, and FLC (Fig. 18). These potential binding sites for AtPIN1 may or may not be accessible to AtPIN1 protein, depending on their specific location within each protein. Due to the substrate specificity of AtPIN1, phosphorylation of these potential sites has to be done by certain kinases before they can be recognized by AtPIN1. It would be interesting to determine whether the Ser/Thr-Pro motifs present in these proteins are functionally regulated by AtPIN1 in controlling flowering time, floral development or other developmental processes. Another issue that needs to be resolved, however, is the significance of *SVP* in regulating *AtPIN1* expression.

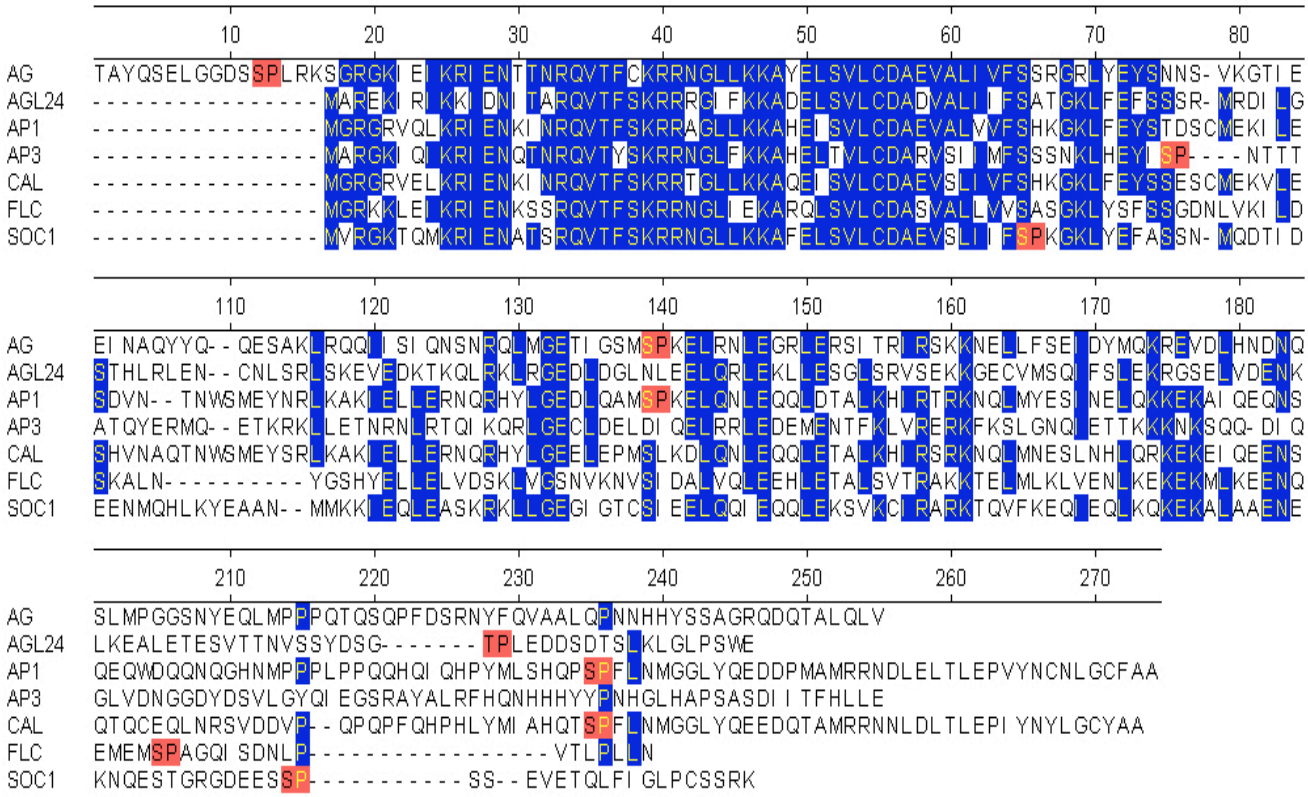

Figure 18. Ser/Thr-Pro motifs in MADS-box transcription factors. Amino acid sequences of a few MADS-box proteins that are involved in flowering or floral development, and contain Ser/Thr-Pro motifs were aligned. Residues that were identical or similar to the consensus were shaded in blue, and Ser/Thr-Pro motifs were shaded in red.

Multiple alignment of amino acid sequences of *Arabidopsis* PIN1 (AtPIN1) with its homologs from *E. coli* (EcPIN1), *Drosophila* (DmPIN1), human (HsPIN1), apple (MdPIN1), and yeast (ScESS1) shows AtPIN1 contains the well conserved PPIase domain, but lacks the WW domain that is conserved in all non-plant eukaryotes. Despite of this difference in structure from its non-plant homologs, its substrate specificity towards phosphorylated Ser/Thr residues preceding proline (pSer/Thr-Pro) remains intact (Landrieu et al., 2000). One of the functions of *PIN1* in mammals is to transcriptionally control the cell cycle arrest genes, $p21^{Cip1}$ and $p27^{Kip1}$ (Brenkman et al., 2008; Wulf et al., 2002). The two genes, $p21^{Cip1}$ and $p27^{Kip1}$, belong to the Cip/Kip family of CDK inhibitors (Pavletich, 1999), and have been suggested to control the G1/S and G2/M transitions by forming inhibitory complexes with different cyclin/CDK complexes (Nakayama and Nakayama, 1998).

In *Arabidopsis*, genome sequence analysis has identified seven genes that show similarity to $p27^{Kip1}$ in sequence and were designated as Kip-related proteins 1 – 7 (KRP1 – KRP7) (De Veylder et al., 2001; Lui et al., 2000; Wang et al., 1997; Zhou et al., 2002). In an attempt to check whether a similar regulatory relationship between *AtPIN1* and *KRPs* is conserved in *Arabidopsis*, as that between *PIN1* and $p27^{Kip1}$ in mammals, molecular analysis of *KRP1* and *KRP2* in various mutants under different conditions and treatments was performed. The expression analysis shows that the expression of *KRP1* and

KRP2 may be modulated by the autonomous and vernalization pathway, while *KRP1* may also be regulated by the GA pathway.

Chapter 5 Conclusion

The timing of floral transitions from vegetative phase to reproductive phase in response to multiple endogenous and environmental signals represents one of the most crucial decisions for plants to make in order to achieve reproductive success. Multiple endogenous and environmental signals are perceived, transmitted, and integrated by multiple flowering pathways in plants to ensure that proper choices are made.

In *Arabidopsis,* SVP has been proposed as a central repressor of flowering. Recent studies have shown that it mainly responds to internal signals and functions by interacting with FLC to control the transcription of two floral pathway integrators, *SOC1* and *FT*. In a search for novel target genes of *SVP* in control of flowering, we have identified *AtPIN1* that was transcriptionally regulated by SVP, and it promoted flowering. Further characterization of the gene revealed that the function of *AtPIN1* as a flowering promoter depended on SOC1 and AGL24 activity, and this interaction between AtPIN1 and SOC1/AGL24 occurred at post-transcriptional level. Our evidence supports a model that, as an enzyme that catalyzes *cis*/*trans* conformational switches, AtPIN1 may bind to SOC1 and AGL24 and facilitate their conformational changes, leading to accumulation of specific conformations of the two proteins

to promote flowering. This would be a novel mechanism that controls flowering at post-transcriptional level and exposes the complexity in the flowering network.

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